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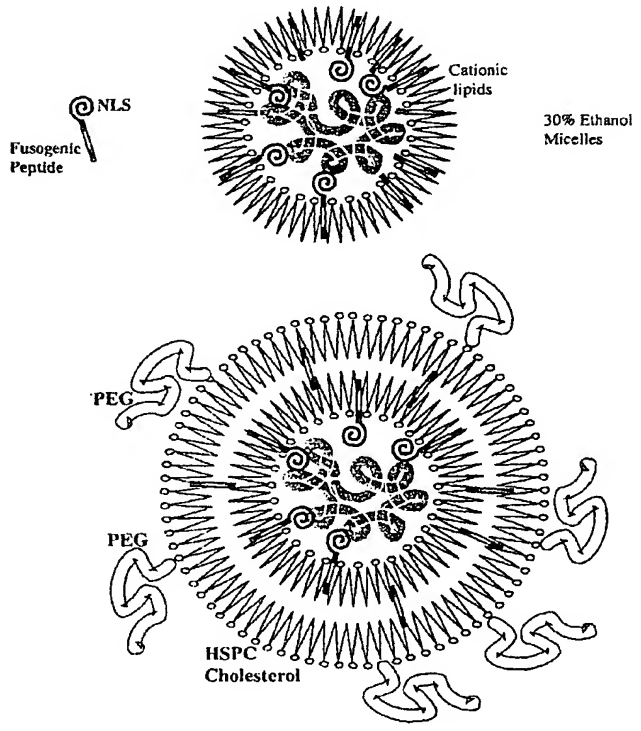
(54) Title: ENCAPSULATION OF PLASMID DNA (LIPOGENES™) AND THERAPEUTIC AGENTS WITH NUCLEAR LOCALIZATION SIGNAL/FUSOGENIC PEPTIDE CONJUGATES INTO TARGETED LIPOSOME COMPLEXES



(57) Abstract: A method is disclosed for encapsulating plasmids, oligonucleotides or negatively-charged drugs into liposomes having a different lipid composition between their inner and outer membrane bilayers and able to reach primary tumors and their metastases after intravenous injection to animals and humans. The formulation method includes complex formation between DNA with cationic lipid molecules and fusogenic/NLS peptide conjugates composed of a hydrophobic chain of about 10-20 amino acids and also containing four or more histidine residues or NLS at their one end. The encapsulated molecules display therapeutic efficacy in eradicating a variety of solid human tumors including but not limited to breast carcinoma and prostate carcinoma. Combination of the plasmids, oligonucleotides or negatively-charged drugs with other anti-neoplastic drugs (the positively-charged cis-platin, doxorubicin) encapsulated into liposomes are of therapeutic value. Also of therapeutic value in cancer eradication are combinations of encapsulated the plasmids, oligonucleotides or negatively-charged drugs with HSV-tk plus encapsulated ganciclovir.

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ENCAPSULATION OF PLASMID DNA (LIPOGENES™) AND THERAPEUTIC AGENTS WITH NUCLEAR LOCALIZATION SIGNAL/FUSOGENIC PEPTIDE CONJUGATES INTO TARGETED LIPOSOME COMPLEXES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 60/210,925 filed June 9, 2000. The contents of this application is hereby incorporated by reference into the present disclosure.

FIELD OF THE INVENTION

The present invention relates to the field of gene therapy and is specifically directed toward methods for producing peptide-lipid-polynucleotide complexes suitable for delivery of polynucleotides to a subject. The peptide-lipid-polynucleotide complexes so produced are useful in a subject for inhibiting the progression of neoplastic disease.

BACKGROUND OF THE INVENTION

Throughout this application various publications, patents and published patent specifications are referenced by author and date or by an identifying patent number. Full bibliographical citations for the publications are provided immediately preceding the claims. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Gene therapy is a newly emerging field of biomedical research that holds great promise for the treatment of both acute and chronic diseases and has the potential to bring a revolutionary era to molecular medicine. However, despite numerous preclinical and clinical studies, routine use of gene therapy for the treatment of human disease has not yet been perfected. It remains an important unmet need of gene therapy to create gene delivery systems that effectively target specific cells of interest in a subject while controlling harmful side effects.

Gene therapy is aimed at introducing therapeutically important genes into somatic cells of patients. Diseases already shown to be amenable to therapy with gene transfer in clinical trials include, cancer (melanoma, breast, lymphoma, head and neck, ovarian, colon, prostate, brain, chronic myelogenous leukemia, non-small cell lung, lung adenocarcinoma, colorectal, neuroblastoma, glioma, glioblastoma, astrocytoma, and others), AIDS, cystic fibrosis, adenosine deaminase deficiency, cardiovascular diseases (restenosis, familial hypercholesterolemia, peripheral artery disease), Gaucher disease, α1-antitrypsin deficiency, rheumatoid arthritis and others. Human diseases expected to be the object of clinical trials include hemophilia A and B, Parkinson's disease, ocular diseases, xeroderma pigmentosum, high blood pressure, obesity. ADA deficiency was the disease successfully treated by the first human "gene transfer" experiment conducted by Kenneth Culver in 1990. See, Culver, K.W. (1996) in: Gene Therapy: A Primer for Physicians, Second Ed., Mary Ann Liebert, Inc. Publ, New York, pp. 1-198.

The primary goals of gene therapy are to repair or replace mutated genes, regulate gene expression and signal transduction, manipulate the immune system, or target malignant and other cells for destruction. See, Anderson, W.F. (1992) *Science 256*:808-813; Lasic, D. (1997) in: Liposomes in Gene Delivery, CRC Press, pp. 1-295; Boulikas, T. (1998) *Gene Ther. Mol. Biol. 1*:1-172; Martin, F. and Boulikas, T. (1998) *Gene Ther. Mol. Biol. 1*:173-214; Ross, G. et al. (1996) *Hum. Gene Ther. 7*:1781-1790.

Human cancer presents a particular disease condition for which effective gene therapy methods would provide a particularly useful clinical benefit. Gene therapy concepts for treatment of such diseases include stimulation of immune responses as well as manipulation of a variety of alternative cellular functions that affect the malignant phenotype. Although many human tumors are non or weakly immunogenic, the immune system can be reinforced and instructed to eliminate cancer cells after transduction of a patient's cells *ex vivo* with the cytokine genes GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, and TNF-α, followed by cell vaccination of the patient (*e.g.* intradermally) to potentiate T-lymphocyte-mediated antitumor effects (cancer immunotherapy). DNA vaccination with genes encoding tumor antigens and immunotherapy with synthetic tumor peptide vaccines are further

developments that are currently being tested. The genes used for cancer gene therapy in human clinical trials include a number of tumor suppressor genes (p53, RB, BRCA1, E1A), antisense oncogenes (antisense *c-fos, c-myc, K-ras),* and suicide genes (HSV-tk, in combination with ganciclovir, cytosine deaminase in combination with 5-fluorocytosine). Other important genes that have been proposed for cancer gene therapy include bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I, VEGF, angiostatin, CFTR, LDL-R, TGF-β, and leptin. One major hurdle preventing successful implementation of these gene therapies is the difficulty of efficiently delivering an effective dose of polynucleotides to the site of the tumor. Thus, gene delivery systems with enhanced transfection capabilities would be highly advantageous.

A number of different vector technologies and gene delivery methods have been proposed and tested for delivering genes *in vivo,* including viral vectors and various nucleic acid encapsulation techniques. Alternative viral delivery vehicles for genes include murine retroviruses, recombinant adenoviral vectors, adeno-associated virus, HSV, EBV, HIV vectors, and baculovirus. Nonviral gene delivery methods use cationic or neutral liposomes, direct injection of plasmid DNA, and polymers. Various strategies to enhance efficiency of gene transfer have been tested such as fusogenic peptides in combination with liposomes or polymers to enhance the release of plasmid DNA from endosomes.

Each of the various gene delivery techniques has been found to possess different strengths and weaknesses. Recombinant retroviruses stably integrate into the chromosome but require host DNA synthesis to insert. Adenoviruses can infect non-dividing cells but cause immune reactions leading to the elimination of therapeutically transduced cells. Adeno-associated virus (AAV) is not pathogenic and does not elicit immune responses but new production strategies are required to obtain high AAV titers for preclinical and clinical studies. Wild-type AAVs integrate into chromosome 19, whereas recombinant AAVs are deprived of site-specific integration and may also persist episomally.

Herpes Simplex Virus (HSV) vectors can infect non-replicating cells, such as neuronal cells, and has a high payload capacity for foreign DNA but inflict cytotoxic effects. It seems that each delivery system will be developed independently of the

others and that each will demonstrate strengths and weaknesses for certain applications. At present, retroviruses are most commonly used in human clinical trials, followed by adenoviruses, cationic liposomes and AAV.

As the challenges of perfecting gene therapy techniques have become apparent, a variety of additional delivery systems have been proposed to circumvent the difficulties observed with standard technologies. For example, cell-based gene delivery using polymer-encapsulated syngeneic or allogeneic cells implanted into a tissue of a patient can be used to secrete therapeutic proteins. This method is being tested in trials for amyotrophic lateral sclerosis using the ciliary neurotrophic factor gene, and may be extended to Factor VIII and IX for hemophilia, interleukin genes, dopamine-secreting cells to treat Parkinson's disease, nerve growth factor for Alzheimer's disease and other diseases. Other techniques under development include, vectors with the Cre-LoxP recombinase system to rid transfected cells of undesirable viral DNA sequences, use of tissue-specific promoters to express a gene in a particular cell type, or use of ligands recognizing cell surface molecules to direct gene vehicles to a particular cell type.

Additional methods that have been proposed for improving the efficacy of gene therapy technologies include designing p53 "gene bombs" that explode into tumor cells, exploiting the HIV-1 virus to engineer vectors for gene transfer, combining viruses with polymers or cationic lipids to improve gene transfer, the attachment of nuclear localization signal peptides to oligonucleotides to direct genes to nuclei, and the development of molecular switch systems allowing genes to be turned on or off at will. Nevertheless, because of the wide range of disease conditions for which gene therapies are required, and the complexities of developing treatments for such diseases, there remains a need for improved techniques for performing gene therapy. The present invention provides methods and compositions for addressing these issues.

DISCLOSURE OF THE INVENTION

A method is disclosed for encapsulating DNA and negatively charged drugs into liposomes having a different lipid composition between their inner and outer membrane bilayers. The liposomes are able to reach primary tumors and their

metastases after intravenous injection to animals and humans. The method includes micelle formation between DNA with a mixture of cationic lipid and peptide molecules at molar ratios to nearly neutralization ratios in 10-90% ethanol; the cationic peptides specify nuclear localization and have a hydrophobic moiety endowed with membrane fusion to improve entrance across the cell membrane of the complex. These peptides insert with their cationic portion directed toward condensed DNA and their hydrophobic chain buried together with the hydrophobic chains of the lipids in the micelle membrane monolayer. The DNA/lipid/peptide micelles are converted into liposomes by mixing with pre-made liposomes or lipids followed by dilution in aqueous solutions and dialysis to remove the ethanol and allow liposome formation and extrusion through membranes to a diameter below 160 nm entrapping and encapsulating DNA with a very high yield. The encapsulated DNA has a high therapeutic efficacy in eradicating a variety of solid human tumors including, but not limited to, breast carcinoma and prostate carcinoma. A plasmid is constructed with DNA carrying anticancer genes including, but not limited to p53, RB, BRCA1, E1A, bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, angiostatin, oncostatin, endostatin, GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, TNF-α, HSV-tk (in combination with ganciclovir), *E. coli* cytosine deaminase (in combination with 5-fluorocytosine) and is combined with encapsulated cisplatin or with other similarly systemically delivered antineoplastic drugs to suppress cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the structure of the cancer targeted liposome complex.

FIG. 2 illustrates the results of plasmid DNA condensation with various agents as well as various formulation of cationic liposomes in affecting the level of expression of the reporter beta-galactosidase gene after transfection of K562 human erythroleukemia cell cultures.

FIG 3 illustrates tumor targeting in SCID mice. FIG 3A shows a SCID mouse with a large and small human breast tumor before and after staining with X-Gal to test the expression of the transferred gene. Both tumors turn dark blue. The intensity of the blue color is proportional to the expression of the beta-galactosidase gene.

FIG 3B shows that in the initial staining of the small tumor, the skin and the intestines at the injection area are the first organs to turn blue. FIG 3C is a view of the back of the animal. The two tumors are clearly visible after removal of the skin (top). Dark staining of the small tumor and light blue staining of the large tumor is evident at an initial stage of staining (bottom). FIG 3D is a view of the front side of the animal. The two tumors are clearly visible after removal of the skin. On the figure to the bottom the dark staining of both tumors is evident at a later stage during staining.

FIG 3E shows the front (top) and rear (bottom) higher magnification view of the dark staining of both tumors at a later stage during staining. Staining of the vascular system around the small tumor can also be seen (bottom).

BRIEF DESCRIPTION OF THE TABLES

Table 1 is a list of molecules able to form micelles.

Table 2 lists several fusogenic peptides and describes their properties, along with a reference.

Table 3 lists simple Nuclear Localization Signal (NLS) peptides.

Table 4 shows a list of "bipartite" or "split" NLS peptides.

Table 5 lists "nonpositive NLS" peptides lacking clusters of arginines/lysines.

Table 6 lists peptides with nucleolar localization signals (NoLS).

Table 7 lists peptides having karyophilic clusters on non-membrane protein kinases.

Table 8 lists peptide nuclear localization signals on DNA repair proteins.

Table 9 lists NLS peptides in transcription factors.

Table 10 lists NLS peptides in other nuclear proteins.

MODES FOR CARRYING OUT THE INVENTION

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA. These methods are described in the following

publications. See, *e.g.*, Sambrook, et al. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F.M. Ausubel, et al. eds., (1987); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR: A PRACTICAL APPROACH, M. MacPherson, et al., IRL Press at Oxford University Press (1991); PCR 2: A PRACTICAL APPROACH, MacPherson et al., eds. (1995); ANTIBODIES, A LABORATORY MANUAL, Harlow and Lane, eds. (1988); and ANIMAL CELL CULTURE, R.I. Freshney, ed. (1987).

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes, for example, single-, double-stranded and triple helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular polypeptide or protein after being transcribed and translated.

A "gene product" refers to the amino acid (*e.g.*, peptide or polypeptide) generated when a gene is transcribed and translated.

The following abbreviations are used herein: **DDAB**: dimethyldioctadecyl ammonium bromide (same as N,N-distearyl-N,N-dimethylammonium bromide); **DODAC**: N,N-dioleyl-N,N-dimethylammonium chloride; DODAP: 1,2-dioleoyl-3-dimethylammonium propane; **DMRIE**: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide; **DMTAP**: 1,2-dimyristoyl-3-trimethylammonium propane; **DOGS**: Dioctadecylamidoglycylspermine; **DOTAP** (same as **DOTMA**): N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; **DOSPA**: N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethyl ammonium trifluoroacetate; **DPTAP**: 1,2- dipalmitoyl-3-trimethylammonium propane; **DSTAP**: 1,2-disteroyl-3-trimethylammonium propane; DOPE, 1,2-sn-dioleoylphoshatidylethanolamine; **DC-Chol**, 3β-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol. See, Gao et al., *Biochem. Biophys. Res. Comm. 179*:280-285 (1991).

As used herein, the term "pharmaceutically acceptable anion" refers to anions of organic and inorganic acids that provide non-toxic salts in pharmaceutical preparations. Examples of such anions include the halides anions, chloride, bromide, and iodide, inorganic anions such as sulfate, phosphate, and nitrate, and organic anions. Organic anions may be derived from simple organic acids, such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic, acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methane sulfonic acid, ethane sulfonic acid, p-toluenesulfonic acid, and the like. The preparation of pharmaceutically acceptable salts is described in Berge, et al., *J. Pharm. Sci. 66*:1-19 (1977), incorporated herein by reference.

Physiologically acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low

molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA: sugar alcohols such as mannitol or sorbitol; salt-forming counter ions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG). PEG molecules also contain a fusogenic peptide with an attached Nuclear Localization Signal (NLS) covalently linked to the end of the PEG molecule.

The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at physiological pH. Such lipids include, but are not limited to, DDAB, DMRIE, DODAC, DOGS, DOTAP, DOSPA and DC-Chol. Additionally, a number of commercial preparations of cationic lipids are available that can be used in the present invention. These include, for example, LIPOFECTIN (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison, Wis., USA).

This invention further provides a number of methods for producing micelles with entrapped therapeutic drugs. The method is particularly useful to produce micelles of drugs or compositions having a net overall negative charge, *e.g.*, DNA, RNA or negatively charged small molecules. For example, the DNA can be comprised within a plasmid vector and encode for a therapeutic protein, *e.g.*, wild-type p53, HSV-tk, p21, Bax, Bad, IL-2, IL-12, GM-CSF, angiostatin, endostatin and oncostatin. In one embodiment, the method requires combining an effective amount of the therapeutic agent with an effective amount of cationic lipids. Cationic lipids useful in the methods of this invention include, but are not limited to, DDAB, dimethyldioctadecyl ammonium bromide; DMRIE: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide; DMTAP: 1,2-dimyristoyl-3-trimethylammonium propane; DOGS: Dioctadecylamidoglycylspermine; DOTAP (same as DOTMA): N-(1-(2,3-

dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DPTAP: 1,2-dipalmitoyl-3-trimethylammonium propane; DSTAP: 1,2-disteroyl-3-trimethylammonium propane.

In one aspect, a ratio of from about 30 to about 90% of phosphates contained within the negatively charged therapeutic agent are neutralized by positive charges on lipid molecules (negative charges are in excess) to form an electrostatic micelle complex in an effective concentration of ethanol. In one aspect, the ethanol solution is from about 20% to about 80% ethanol. In a further aspect, the ethanol concentration is about 30%. The ethanol/cationic lipid/therapeutic agent complex is then combined with an effective amount of a fusogenic-karyophilic peptide conjugate. In one aspect, an effective amount of the conjugate is a ratio range from about 0.0 to about 0.3 (positive charges on peptide to negative charges on phosphate groups) to neutralize the majority of the remaining negative charges on the phosphate groups of the therapeutic agents thereby leading to an almost complete neutralization of the complex. The optimal conditions give to the complex a slightly negative charge. However, when the positive charges on cationic lipids exceed the negative charges on the DNA, the excess of positive charges are neutralized by DPPG (dipalmitoyl phosphatidyl glycerol) and its derivatives, or by other anionic lipid molecules in the final micelle complex.

In an alternative embodiment, the above methods can be modified by addition of DNA condensing agents selected from spermine, spermidine, and magnesium or other divalent metal ions neutralizing a certain percentage (1-20%) of phosphate groups.

In a further embodiment, the cationic lipids are combined with an effective amount of fusogenic lipid DOPE at various molar ratios for example, in a molar ratio of from about 1:1 cationic lipid:DOPE. In an alternative embodiment, the cationic lipids are combined with an effective amount of a fusogenic/NLS peptide conjugate. Examples of fusogenic/NLS peptide conjugates include, but are not limited to $(KAWLKAF)_3$ (SEQ ID NO:1), GLFKAAAKLLKSLWKLLLKA (SEQ ID NO:2), LLLKAFAKLLKSLWKLLLKA (SEQ ID NO:3), as well as all derivatives of the prototype (Hydrophobic3-Karyophilic1-Hydrophobic2-Karyophilic1)$_{2-3}$ where Hydrophobic is any of the A, I, L, V, P, G, W, F and

Karyophilic is any of the K, R, or H, containing a positively-charged residue every 3rd or 4th amino acid, which form alpha helices and direct a net positive charge to the same direction of the helix. Additional examples include but are not limited to GLFKAIAGFIKNGWKGMIDGGGYC (SEQ ID NO:4) from influenza virus hemagglutinin HA-2; YGRKKRRQRRR (SEQ ID NO:5) from TAT of HIV; MSGTFGGILAGLIGLL(K/R/H)$_{1-6}$ (SEQ ID NO:6), derived from the N-terminal region of the S protein of duck hepatitis B virus, but with the addition of one to six positively-charged lysine, arginine or histidine residues, and combinations of these, able to interact directly with the phosphate groups of plasmid or oligonucleotide DNA, compensating for part of the positive charges provided by the cationic lipids. GAAIGLAWIPYFGPAA (SEQ ID NO:7) is derived from the fusogenic peptide of the Ebola virus transmembrane protein; residues 53-70 (C-terminal helix) of apolipoprotein (apo) AII peptide; the 23-residue fusogenic N-terminal peptide of HIV-1 transmembrane glycoprotein gp41; the 29-42-residue fragment from Alzheimer's β-amyloid peptide; the fusion peptide and N-terminal heptad repeat of Sendai virus; the 56-68 helical segment of lecithin cholesterol acyltransferase. Included within these embodiments are shorter versions of these peptides, that are known to induce fusion of unilamellar lipid vesicles or all that are similarly derivatized with the addition of one to six positively-charged lysine, arginine or histidine residues (K/R/H)$_{1-6}$ able to interact directly with the phosphate groups of plasmid or oligonucleotide DNA, compensating for part of the positive charges provided by the cationic lipids. The fusogenic peptides in the fusogenic/NLS conjugates represent hydrophobic amino acid stretches, and smaller fragments of these peptide sequences, that include all signal peptide sequences used in membrane or secreted proteins that insert into the endoplasmic reticulum. Alternatively, the conjugates represent transmembrane domains and smaller fragments of these peptide sequences.

In one aspect of the invention, the NLS peptide component in fusogenic/NLS peptide conjugates is derived from the fusogenic hydrophobic peptides. However, there is an addition of 5-6 amino acid karyophilic Nuclear Localization Signals (NLS) derived from a number of known NLS peptides, as well as from searches of the nuclear protein databases, for stretches of five or more

karyophilic amino acid stretches in proteins containing at least four positively-charged amino aids flanked by a proline (P) or glycine (G). Examples of NLS peptides are shown in Tables 1-8. The NLS peptide component in fusogenic/NLS peptide conjugates are synthetic peptides containing the above said NLS, but further modified by additional K, R, H residues at the central part of the peptide or with P or G at the N- or C-terminus.

In a further aspect, the fusogenic/NLS peptide conjugates are derived from the said fusogenic hydrophobic peptides but with the addition of a stretch of H_{4-6} (four to six histidine residues) in the place of NLS. Micelle formation takes place at pH 5-6 where histidyl residues are positively charged but lose their charge at the nearly neutral pH of the biological fluids, thus releasing the plasmid or oligonucleotide DNA from their electrostatic interaction.

The fusogenic peptide/NLS peptide conjugates are linked to each other with a short amino acid stretch representing an endogenous protease cleavage site.

In a preferred aspect of the invention, the structure of the preferred prototype fusogenic/NLS peptide conjugate used in this invention is: P**KKRR**GPSP$(L/A/I)_{12-20}$ (SEQ ID NO:8), where $(L/A/I)_{12-20}$ is a stretch of 12-20 hydrophobic amino acids containing A, L, I, Y, W, F and other hydrophobic amino acids.

The micelles made by the above methods are further provided by this invention by conversion into liposomes. An effective amount of liposomes (diameter from about 80 to about 160 nm), or of a lipid solution composed of cholesterol (from about 10% to about 50%), neutral phospholipid such as hydrogenated soy phosphatidylcholine (HSPC) (from about 40% to about 90%), and the derivatized vesicle-forming lipid PEG-DSPE (distearoylphosphatidyl ethanolamine) from about 1-to about 7 mole percent, is added to the micelle solution.

In a specific embodiment, the liposomes are composed of vesicle-forming lipids and between from about 1 to about 7 mole percent of distearoylphosphatidyl ethanolamine (DSPE) derivatized with a polyethyleneglycol. The composition of claim 20, wherein the polyethyleneglycol has a molecular weight is between about 1,000 to 5,000 daltons. Micelles are converted into liposomes with a concomitant decrease of the ethanol concentration which can be accomplished by removal of the

ethanol by dialysis of the liposome complexes through permeable membranes or reduced to a diameter of 80-160 nm by extrusion through membranes.

Liposome encapsulated therapeutic agents produced by the above methods are further provided by this invention.

Also provided herein is a method for delivering a therapeutic agent such as plasmid DNA or oligonucleotides to a tissue cell *in vivo* by intravenous, or other type of injection of the micelles or liposomes. This method specifically targets a primary tumor and the metastases by the long circulating time of the micelle or liposome complex because of the exposure of PEG chains on its surface, its small size (80-160 nm) and the decrease in hydrostatic pressure in the solid tumor from the center to its periphery supporting a preferential extravasation through the tumor vasculature to the extracellular space in tumors. A method for delivering plasmid or oligonucleotide DNA across the cell membrane barrier of the tumors using the micelle or liposome complexes described herein is capable because of the presence of the fusogenic peptides in the complex. In particular, a method for delivering plasmid or oligonucleotide DNA to the liver, spleen and bone marrow after intravenous injection of the complexes is provided. Further provided is a method for delivering therapeutic genes to the liver, spleen and bone marrow of cancer and noncancer patients including but not limited to, factor VIII or IX for the therapy of hemophilias, multidrug resistance, cytokine genes for cancer immunotherapy, genes for the alleviation of pain, genes for the alleviation of diabetes and genes that can be introduced to liver, spleen and bone marrow tissue, to produce a secreted form of a therapeutic protein.

The disclosed therapies also provide methods for reducing tumor size by combining the encapsulated plasmid DNA carrying one or more anticancer genes selected from the group consisting of p53, RB, BRCA1, E1A, bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, angiostatin, oncostatin, endostatin, GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, TNF-α, HSV-tk (in combination with ganciclovir), *E. coli* cytosine deaminase (in combination with 5-fluorocytosine) with encapsulated antisense oligonucleotides (antisense c-fos, c-myc, K-ras), ribozymes or triplex-forming oligonucleotides directed against genes that control the cell cycle or signaling pathways. These methods can be modified by combining the

encapsulated plasmid DNA carrying one or more anticancer genes of with encapsulated or free antineoplastic drugs, consisting of the group of adriamycin, angiostatin, azathioprine, bleomycin, busulfane, camptothecin, carboplatin, carmustine, chlorambucile, chlormethamine, chloroquinoxaline sulfonamide, cisplatin, cyclophosphamide, cycloplatam, cytarabine, dacarbazine, dactinomycin, daunorubicin, didox, doxorubicin, endostatin, enloplatin, estramustine, etoposide, extramustinephosphat, flucytosine, fluorodeoxyuridine, fluorouracil, gallium nitrate, hydroxyurea, idoxuridine, interferons, interleukins, leuprolide, lobaplatin, lomustine, mannomustine, mechlorethamine, mechlorethaminoxide, melphalan, mercaptopurine, methotrexate, mithramycin, mitobronitole, mitomycin, mycophenolic acid, nocodazole, oncostatin, oxaliplatin, paclitaxel, pentamustine, platinum-triamine complex, plicamycin, prednisolone, prednisone, procarbazine, protein kinase C inhibitors, puromycine, semustine, signal transduction inhibitors, spiroplatin, streptozotocine, stromelysin inhibitors, taxol, tegafur, telomerase inhibitors, teniposide, thalidomide, thiamiprine, thioguanine, thiotepa, tiamiprine, tretamine, triaziquone, trifosfamide, tyrosine kinase inhibitors, uramustine, vidarabine, vinblastine, vinca alcaloids, vincristine, vindesine, vorozole, zeniplatin, zeniplatin, and zinostatin.

The following examples are intended to illustrate, but not limit the invention.

Liposome Composition

Liposomes are microscopic vesicles consisting of concentric lipid bilayers. Structurally, liposomes range in size and shape from long tubes to spheres, with dimensions from a few hundred Angstroms to fractions of a millimeter. Vesicle-forming lipids are selected to achieve a specified degree of fluidity or rigidity of the final complex providing the lipid composition of the outer layer. These are neutral (cholesterol) or bipolar and include phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (SM) and other type of bipolar lipids including but not limited to dioleoylphosphatidylethanolamine (DOPE), with a hydrocarbon chain length in the range of 14-22, and saturated or with one or more double C=C bonds. Examples of lipids capable of producing a stable liposome, alone, or in combination with other

lipid components are phospholipids, such as hydrogenated soy phosphatidylcholine (HSPC), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, distearoylphosphatidylethanolamine (DSPE), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE) and dioleoylphosphatidylethanolamine 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (DOPE-mal). Additional non-phosphorous containing lipids that can become incorporated into liposomes include stearylamine, dodecylamine, hexadecylamine, isopropyl myristate, triethanolamine-lauryl sulfate, alkyl-aryl sulfate, acetyl palmitate, glycerol ricinoleate, hexadecyl stereate, amphoteric acrylic polymers, polyethyloxylated fatty acid amides, and the cationic lipids mentioned above (DDAB, DODAC, DMRIE, DMTAP, DOGS, DOTAP (DOTMA), DOSPA, DPTAP, DSTAP, DC-Chol). Negatively charged lipids include phosphatidic acid (PA), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylglycerol and (DOPG), dicetylphosphate that are able to form vesicles. Preferred lipids for use in the present invention are cholesterol, hydrogenated soy phosphatidylcholine (HSPC) and, the derivatized vesicle-forming lipid PEG-DSPE.

Typically, liposomes can be divided into three categories based on their overall size and the nature of the lamellar structure. The three classifications, as developed by the New York Academy Sciences Meeting, "Liposomes and Their Use in Biology and Medicine," December 1977, are multi-lamellar vesicles (MLVs), small uni-lamellar vesicles (SUVs) and large uni-lamellar vesicles (LUVs).

SUVs range in diameter from approximately 20 to 50 nm and consist of a single lipid bilayer surrounding an aqueous compartment. Unilamellar vesicles can also be prepared in sizes from about 50 nm to 600 nm in diameter. While unilamellar are single compartmental vesicles of fairly uniform size, MLVs vary greatly in size up to 10,000 nm, or thereabouts, are multi-compartmental in their structure and contain more than one bilayer. LUV liposomes are so named because of their large diameter that ranges from about 600 nm to 30,000 nm; they can contain more than one bilayer.

Liposomes may be prepared by a number of methods not all of which produce the three different types of liposomes. For example, ultrasonic dispersion by means of immersing a metal probe directly into a suspension of MLVs is a common way for preparing SUVs.

Preparing liposomes of the MLV class usually involves dissolving the lipids in an appropriate organic solvent and then removing the solvent under a gas or air stream. This leaves behind a thin film of dry lipid on the surface of the container. An aqueous solution is then introduced into the container with shaking, in order to free lipid material from the sides of the container. This process disperses the lipid, causing it to form into lipid aggregates or liposomes. Liposomes of the LUV variety may be made by slow hydration of a thin layer of lipid with distilled water or an aqueous solution of some sort. Alternatively, liposomes may be prepared by lyophilization. This process comprises drying a solution of lipids to a film under a stream of nitrogen. This film is then dissolved in a volatile solvent, frozen, and placed on a lyophilization apparatus to remove the solvent. To prepare a pharmaceutical formulation containing a drug, a solution of the drug is added to the lyophilized lipids, whereupon liposomes are formed.

Preparing Cationic Liposome/Cationic Peptide/Nucleic Acid Micelles

Cationic lipids, with the exception of sphingosine and some lipids in primitive life forms, do not occur in nature. The present invention uses single-chain amphiphiles which are chloride and bromide salts of the alkyltrimethylammonium surfactants including but not limited to C12 and C16 chains abbreviated DDAB (same as DODAB) or CTAB. The molecular geometry of these molecules determines the critical micelle concentration (ratio between free monomers in solution and molecules in micelles). Lipid exchange between the two states is a highly dynamic process; phospholipids have critical micelle concentration values below 10^{-8} M and are more stable in liposomes; however, single chain detergents, such as stearylamine, may emerge from the liposome membrane upon dilution or intravenous injection in milliseconds (Lasic, 1997).

Cationic lipids include, but are not limited to, DDAB: dimethyldioctadecyl ammonium bromide (same as N,N-distearyl-N,N-dimethylammonium bromide);

DMRIE: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide; DODAC: N,N-dioleyl-N,N-dimethylammonium chloride; DMTAP: 1,2-dimyristoyl-3-trimethylammonium propane; DODAP: 1,2-dioleoyl-3-dimethylammonium propane; DOGS: Dioctadecylamidoglycylspermine; DOTAP (same as DOTMA): N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DOSPA: N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethyl ammonium trifluoroacetate; DPTAP: 1,2- dipalmitoyl-3-trimethylammonium propane; DSTAP: 1,2-disteroyl-3-trimethylammonium propane; DC-Chol, 3β-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol.

Lipid-based vectors used in gene transfer have been formulated in one of two ways. In one method, the nucleic acid is introduced into preformed liposomes made of mixtures of cationic lipids and neutral lipids. The complexes thus formed have undefined and complicated structures and the transfection efficiency is severely reduced by the presence of serum. Preformed liposomes are commercially available as LIPOFECTIN and LIPOFECTAMINE. The second method involves the formation of DNA complexes with mono- or poly-cationic lipids without the presence of a neutral lipid. These complexes are prepared in the presence of ethanol and are not stable in water. Additionally, these complexes are adversely affected by serum (see, Behr, *Acc. Chem. Res. 26*:274-78 (1993)). An example of a commercially available poly-cationic lipid is TRANSFECTAM. Other efforts to encapsulate DNA in lipid-based formulations have not overcome these problems (see, Szoka et al., *Ann. Rev. Biophys. Bioeng. 9*:467 (1980); and Deamer, U.S. Patent No. 4,515,736).

The nucleotide polymers can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as plasmid DNA. Particularly preferred nucleic acids are plasmids. Single-stranded nucleic acids include antisense oligonucleotides (complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides. In order to increase stability, some single-stranded nucleic acids will preferably have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, for example, phosphorothioate,

phosphorodithioate, phosphoroselenate, methylphosphonate, or O-alkyl phosphotriester linkages.

Encapsulating Cationic Liposome/Cationic Peptide/Nucleic Acid Micelles into Neutral Liposomes

Cationic lipids used with fusogenic peptide/NLS conjugates to provide the inner layer of the particle can be any of a number of substances selected from the group of DDAB, DODAC, DMRIE, DMTAP, DOGS, DOTAP (DOTMA), DOSPA, DPTAP, DSTAP, DC-Chol. The cationic lipid is combined with DOPE. In one group of embodiments, the preferred cationic lipid is DDAB:DOPE 1:1.

Neutral lipids used herein to provide the outer layer of the particles can be any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids are selected from a group consisting of diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. In one group of embodiments, lipids containing saturated, mono-, or di-unsaturated fatty acids with carbon chain lengths in the range of C14 to C22 are preferred. In general, less saturated lipids are more easily sized, particularly when the liposomes must be sized below about 0.16 microns, for purposes of filter sterilization. Consideration of liposome size, rigidity and stability of the liposomes in the final preparation, its shelf life without leakage of the encapsulated DNA, and stability in the bloodstream generally guide the selection of neutral lipids for providing the outer coating of our gene vehicles. Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In another group of embodiments, lipids with carbon chain lengths in the range of C14 to C22 are used. Preferably, the neutral lipids used in the present invention are hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PEG-distearoylphosphatidyl ethanolamine (DSPE) or PEG-ceramide.

Methods for preparing liposomes

A variety of methods for preparing various liposome forms have been described in several issued patents, for example, U.S. Patent Nos. 4,229,360;

4,224,179; 4,241,046; 4,737,323; 4,078,052; 4,235,871; 4,501,728; and 4,837,028, as well as in the articles Szoka et al., *Ann. Rev. Biophys. Bioeng. 9*:467 (1980) and Hope et al., *Chem. Phys. Lip. 40*:89 (1986). These methods do not produce all three different types of liposomes (MLVs, SUVs, LUVs). For example, ultrasonic dispersion by means of immersing a metal probe directly into a suspension of MLVs is a common way for preparing SUVs.

Preparing liposomes of the MLV class usually involves dissolving the lipids in an appropriate organic solvent and then removing the solvent under a gas or air stream. This leaves behind a thin film of dry lipid on the surface of the container. An aqueous solution is then introduced into the container with shaking, in order to free lipid material from the sides of the container. This process disperses the lipid, causing it to form into lipid aggregates or liposomes. Liposomes of the LUV variety may be made by slow hydration of a thin layer of lipid with distilled water or an aqueous solution of some sort. Alternatively, liposomes may be prepared by lyophilization. This process comprises drying a solution of lipids to a film under a stream of nitrogen. The film is then dissolved in a volatile solvent, frozen, and placed on a lyophilization apparatus to remove the solvent. To prepare a pharmaceutical formulation containing a drug, a solution of the drug is added to the lyophilized lipids, whereupon liposomes are formed.

Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. Preferably, the preformed liposomes are sized to a mean diameter of about 80 to 160 nm (the upper size limit for filter sterilization before *in vivo* administration). Several techniques are available for sizing liposomes to a desired size. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns (50 nm) in size. Extrusion of liposome through a small-pore polycarbonate is our preferred method for reducing liposome sizes to a relatively well-defined size distribution. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

One way used to coat DNA with lipid is by controlled detergent depletion from a cationic lipid/DNA/detergent complex. This method can give complexes

with stability in plasma. Hofland et al. (1996), have prepared such complexes by dialysis of a mixture of DOSPA/DOPE/DNA/octylglucoside.

Pharmaceutical compositions comprising the cationic liposome/nucleic acid complexes of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier.

For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intravenously, intraperitoneally, subcutaneously, intrathecally, injection to the spinal cord, intramuscularly, intraarticularly, portal vein injection, or intratumorally. More preferably, the pharmaceutical compositions are administered intravenously or intratumorally by a bolus injection. In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical "open" or "closed" procedures. The term "topical" means the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, to any surface of the body, nasopharynx, external auditory canal, ocular administration and administration to the surface of any body cavities, inhalation to the lung, genital mucosa and the like.

"Open" procedures are those procedures that include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue.

"Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via insertion of instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

EXAMPLES

Materials and Methods

DDAB, DOPE (dioleoylphosphatidylethanolamine) and most other lipids used here were purchased from Avanti Polar Lipids; PEG-DSPE was from Syngena.

Engineering of plasmid pLF

The pGL3-C (Promega) was cut with XbaI and blunt-end ligated using the Klenow fragment of *E. coli* DNA polymerase. It was then cut with HindIII and the 1689-bp fragment, carrying the luciferase gene, was gel-purified. The pGFP-N1 plasmid (Clontech) was cut with SmaI and HindIII and the 4.7 kb fragment, isolated from an agarose gel, was ligated with the luciferase fragment. JM109 *E. coli* cells were transformed and 20 colonies were selected; about half of them showed the presence of inserts; 8 clones with inserts were cut with BamHI and XhoI to further confirm the presence of the luciferase gene; seven of them were positive.

Radiolabeled plasmid **pLF** was generated by culturing *Escherichia coli* in ^{3}H-thymidine-5'-triphosphate or ^{32}P inorganic phosphate (5 mCi) (Dupont/NEN, Boston, Mass.) and purified using standard techniques as described above.

DLS measurements

A Coulter N4M light scattering instrument was used, at a 90° angle, set at a run time of 200 sec, using 4 to 25 microsec sample time. The scan of the particle size distribution was obtained in 1 ml sample volume using plastic cuvettes, at 20°C and at 0.01 poise viscosity.

In one aspect, this invention provides a method for entrapping DNA into lipids that enhances the content of plasmid per volume unit, and reduces the toxicity of the cationic lipids used to trap plasmid or oligonucleotide DNA. The DNA becomes hidden in the inner membrane bilayer of the final complex. Furthermore, the gene transfer complex is endowed with long circulation time in body fluids and extravasates preferentially into solid tumors and their metastatic foci and nodules. The extravasation occurs through their vasculature at most sites of the human or animal body after intravenous injection of the gene-carrying vehicles. This occurs because of their small size (100-160 nm), their content in neutral to slightly

negatively-charged lipids in their outer membrane bilayers, and their coating with PEG. These gene delivery vehicles are able to cross the cell membrane barrier after they reach the extracellular tumor space because of the presence of fusogenic peptides conjugated with karyophilic peptides. The vehicles assume a certain predefined orientation in the lipid membrane with their positive ends directed toward DNA and their hydrophobic tail buried inside the hydrophobic lipid bilayer. The labile NLS-fusogenic peptide linkage is cleaved after endocytosis and the remaining NLS peptide bound to plasmid DNA aids its nuclear uptake. This occurs especially when non-dividing cells are targeted, such as liver, spleen or bone marrow cells that represent the major sites for extravasation and concentration of these vehicles other than solid tumors.

Organic solvent

A suitable solvent for preparing a micelle from the desired lipid components is ethanol, methanol, or other aliphatic alcohols such as propanol, isopropanol, butanol, tert-butanol, iso-butanol, pentanol and hexanol. Mixtures of two or more solvents may be used in the practice of the invention. It is also to be understood that any solvent that is miscible with an ethanol solution, even in small amounts, can be used to improve micelle formation and its subsequent conversion into liposomes, including chloroform, dichloromethane, diethylether, cyclohexane, cyclopentane, benzene, and toluene.

Cationic lipids

In a further embodiment, the liposome encapsulated DNA described herein further comprises an effective amount of cationic lipids. Cationic lipids have been widely used for gene transfer; a number of clinical trials (34 out of 220 total RAC-approved protocols as of December, 1997) use cationic lipids. Although many cell culture studies have been documented, systemic delivery of genes with cationic lipids *in vivo* has been very limited. All clinical protocols use subcutaneous, intradermal, intratumoral, and intracranial injection as well as intranasal, intrapleural, or aerosol administration but not I.V. delivery, because of the toxicity of the cationic lipids and DOPE (see, Martin and Boulikas, 1998). Liposomes

formulated from DOPE and cationic lipids based on diacyltrimethylammonium propane (dioleoyl-, dimyristoyl-, dipalmitoyl-, disteroyl-trimethylammonium propane or DOTAP, DMTAP, DPTAP, DSTAP, respectively) or DDAB were highly toxic when incubated *in vitro* with phagocytic cells (macrophages and U937 cells), but not towards non-phagocytic T lymphocytes. The rank order of toxicity was DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP; and the toxicity was determined from the effect of the cationic liposomes on the synthesis of nitric oxide (NO) and TNF-α produced by activated macrophages (Filion and Phillips, 1997).

Another aspect to be considered before I.V. injection is undertaken, is that negatively charged serum proteins can interact and cause inactivation of cationic liposomes (Yang and Huang, 1997). Condensing agents used for plasmid delivery including polylysine, transferrin-polylysine, a fifth-generation poly(amidoamine) (PAMAM) dendrimer, poly(ethyleneimine), and several cationic lipids (DOTAP, DC-Chol/DOPE, DOGS/DOPE, and DOTMA/DOPE), were found to activate the complement system to varying extents. Strong complement activation was seen with long-chain polylysines, the dendrimer, poly(ethyleneimine), and DOGS. Modifying the surface of preformed DNA complexes with polyethyleneglycol (Plank et al., 1996) considerably reduced complement activation.

Cationic lipids increase the transfection efficiency by destabilizing the biological membranes, including plasma, endosomal, and lysosomal membranes. Incubation of isolated lysosomes with low concentrations of DOTAP caused a striking increase in free activity of β-galactosidase, and even a release of the enzyme into the medium. This demonstrates that the lysosomal membrane is deeply destabilized by the lipid. The mechanism of destabilization was thought to involve an interaction between cationic liposomes and anionic lipids of the lysosomal membrane, thus allowing a fusion between the lipid bilayers. The process was less pronounced at pH 5 than at pH 7.4, and anionic amphipathic lipids were able to prevent partially this membrane destabilization (Wattiaux et al., 1997).

In contrast to DOTAP and DMRIE that were 100% charged at pH 7.4, DC-CHOL was only about 50% charged as monitored by a pH-sensitive fluorophore. This difference decreases the charge on the external surfaces of the liposomes, and

was proposed to promote an easier dissociation of bilayers containing DC-CHOL from the plasmid DNA, and an increase in release of the DNA-lipid complex into the cytosol from the endosomes (Zuidam and Barenholz, 1997).

Although cationic lipids have been used widely for the delivery of genes, very few studies have used systemic I.V. injection of cationic liposome-plasmid complexes. This is because of the toxicity of the lipid component in animal models, not humans. Administration by I.V. injection of two types of cationic lipids of similar structure, DOTMA and DOTAP, shows that the transfection efficiency is determined mainly by the structure of the cationic lipid and the ratio of cationic lipid to DNA; the luciferase and GFP gene expression in different organs was transient, with a peak level between 4 and 24 hr, dropping to less than 1% of the peak level by day 4 (Song et al., 1997).

A number of different organs *in vivo* can be targeted after liposomal delivery of genes or oligonucleotides. Intravenous injection of cationic liposome-plasmid complexes by tail vein in mice, targeted mainly the lung and to a smaller extent the liver, spleen, heart, kidney and other organs (Zhu et al., 1993). Intraperitoneal injection of a plasmid-liposome complex expressing antisense K-ras RNA in nude mice inoculated i.p. with AsPC-1 pancreatic cancer cells harboring K-ras point mutations and PCR analysis indicated that the injected DNA was delivered to various organs except brain (Aoki et al., 1995).

A number of factors for DOTAP:cholesterol/DNA complex preparation including the DNA:liposome ratio, mild sonication, heating, and extrusion were found to be crucial for improved systemic delivery; maximal gene expression was obtained when a homogeneous population of DNA:liposome complexes between 200 to 450 nm in size were used. Cryo-electron microscopy showed that the DNA was condensed on the interior of invaginated liposomes between two lipid bilayers in these formulations, a factor that was thought to be responsible for the high transfection efficiency *in vivo* and for the broad tissue distribution (Templeton et al., 1997).

Steps to improve liposome-mediated gene delivery to somatic cells include, persistence of the plasmid in blood circulation, port of entry and transport across the cell membrane, release from endosomal compartments into the cytoplasm, nuclear

import by docking through the pore complexes of the nuclear envelope, expression driven by the appropriate promoter/enhancer control elements, and persistence of the plasmid in the nucleus for long periods (Boulikas, 1998a).

Plasmid condensation with spermine

In a further embodiment, the liposome encapsulated DNA described herein is condensed with spermine and/or spermidine. DNA can be presented to cells in culture as a complex with polycations such as polylysine, or basic proteins such as protamine, total histones or specific histone fractions, protamine (Boulikas and Martin, 1997). The interaction of plasmid DNA with protamine sulfate, followed by the addition of DOTAP cationic liposomes, offered a better protection of plasmid DNA against enzymatic digestion. The method gave consistently higher gene expression in mice via tail vein injection as compared with DOTAP/DNA complexes. 50 μg of luciferase-plasmid per mouse gave 20 ng luciferase protein per mg extracted tissue protein in the lung, that was detected as early as 1 h after injection, peaked at 6 h and declined thereafter. Intraportal injection of protamine/DOTAP/DNA led to about a 100-fold decrease in gene expression in the lung as compared with I.V. injection. Endothelial cells were the primary locus of lacZ transgene expression (Li and Huang, 1997). Protamine sulfate enhanced plasmid delivery into several different types of cells *in vitro,* using the monovalent cationic liposomal formulations (DC-Chol and lipofectin). This effect was less pronounced with the multivalent cationic liposome formulation, lipofectamine (Sorgi et al., 1997).

Spermine is found to enhance the transfection efficiency of DNA-cationic liposome complexes in cell culture and in animal studies. This biogenic polyamine at high concentrations caused liposome fusion most likely promoted by the simultaneous interaction of one molecule of spermine (four positively charged amino groups) with the polar head groups of two or more molecules of lipids. At low concentrations (0.03-0.1 mM) it promoted anchorage of the liposome-DNA complex to the surface of cells and enhanced significantly transfection efficiency (Boulikas, unpublished).

The polycations polybrene, protamine, DEAE-dextran, and poly-L-lysine significantly increased the efficiency of adenovirus-mediated gene transfer in cell culture. This was thought to act by neutralizing the negative charges presented by membrane glycoproteins that reduce the efficiency of adenovirus-mediated gene transfer (Arcasoy et al., 1997).

Oligonucleotide transfer

In a further embodiment, the liposome encapsulates oligonucleotide DNA. Encapsulation of oligonucleotides into liposomes increased their therapeutic index, prevented degradation in cultured cells, and in human serum and reduced toxicity to cells (Thierry and Dritschilo, 1992; Capaccioli et al., 1993; Lewis et al., 1996). However, most studies have been performed in cell culture, and very few in animals *in vivo*. There are still an important number of improvements needed before these approaches can move into clinical studies.

Zelphati and Szoka (1997), have found that complexes of fluorescently labeled oligonucleotides with DOTAP liposomes, entered the cell using an endocytic pathway mainly involving uncoated vesicles. Oligonucleotides were redistributed from punctate cytoplasmic regions into the nucleus. This process was independent of acidification of the endosomal vesicles. The nuclear uptake of oligonucleotides depended on several factors, such as charge of the particle, where positively charged complexes were required for enhanced nuclear uptake. DOTAP increased over 100 fold the antisense activity of a specific anti-luciferase oligonucleotide. Physicochemical studies of oligonucleotide-liposome complexes of different cationic lipid compositions indicated that either phosphatidylethanolamine or negative charges on other lipids in the cell membrane are required for efficient fusion with cationic liposome-oligonucleotide complexes to promote entry to the cell (Jaaskelainen et al., 1994).

Similar results were reported by Lappalainen et al. (1997). Digoxigenin-labeled oligodeoxynucleotides (ODNs) complexed with the polycationic DOSPA and the monocationic DDAB (with DOPE as a helper lipid) were taken up by CaSki cells in culture by endocytosis. The nuclear membrane was found to pose a barrier against nuclear import of ODNs that accumulated in the perinuclear area. Although

DOSPA/DOPE liposomes could deliver ODNs into the cytosol, they were unable to mediate nuclear import of ODNs. On the contrary, oligonucleotide-DDAB/DOPE complexes with a net positive charge were released from vesicles into the cytoplasm. It was determined that DDAB/DOPE mediated nuclear import of the oligonucleotides.

DOPE-heme (ferric protoporphyrin IX) conjugates, inserted in cationic lipid particles with DOTAP, protected oligoribonucleotides from degradation in human serum and increased oligoribonucleotide uptake into 2.2.15 human hepatoma cells. The enhancing effect of heme was evident only at a net negative charge in the particles (Takle et al., 1997). Uptake of liposomes labeled with ^{111}In and composed of DC-Chol and DOPE was primarily by liver, with some accumulation in spleen and skin and very little in the lung after I.V. tail injection. Preincubation of cationic liposomes with phosphorothioate oligonucleotide induced a dramatic, yet transient, accumulation of the lipid in lung that gradually redistributed to liver. The mechanism of lung uptake involved entrapment of large aggregates of oligonucleotides within pulmonary capillaries at 15 min post-injection via embolism. Labeled oligonucleotide was localized primarily to phagocytic vacuoles of Kupffer cells at 24 h post-injection. Nuclear uptake of oligonucleotides *in vivo* was not observed (Litzinger et al., 1996).

Polyethylene glycol (PEG)-coated liposomes

In a further embodiment, the liposome encapsulated DNA described herein, further comprise coating of the final complex in step 2 (Fig. 1) with PEG. It is often desirable to conjugate a lipid to a polymer that confers extended half-life, such as polyethylene glycol (PEG). Derivatized lipids that are employed, include PEG-modified DSPE or PEG-ceramide. Addition of PEG components prevents complex aggregation, increases circulation lifetime of particles (liposomes, proteins, other complexes, drugs) and increases the delivery of lipid-nucleic acid complexes to the target tissues. See, Maxfield et al., *Polymer 16*:505-509 (1975); Bailey, F.E. et al., in: Nonionic Surfactants, Schick, M.J., ed., pp. 794-821 (1967); Abuchowski, A. et al., *J. Biol. Chem. 252*:3582-3586 (1977); Abuchowski, A. et al., *Cancer Biochem.*

Biophys. 7:175-186 (1984); Katre, N.V. et al., *Proc. Natl. Acad. Sci. USA* 84:1487-1491 (1987); Goodson, R. et al. *Bio Technology* 8:343-346 (1990).

Conjugation to PEG is reported to have reduced immunogenicity and toxicity. See, Abuchowski et al., *J. Biol. Chem.* 252:3578-3581 (1977). The extent of enhancement of blood circulation time of liposomes, by coating with PEG is described in U.S. Patent No. 5,013,556. Typically, the concentration of the PEG-modified phospholipids, or PEG-ceramide in the complex will be about 1-7%. In a particularly preferred embodiment, the PEG-modified lipid is a PEG-DSPE.

Coating the surface of liposomes with inert materials designed to camouflage the liposome from the body's host defense systems was shown to increase remarkably the plasma longevity of liposomes. The biological paradigm for this "surface modified" sub-branch was the erythrocyte, a cell that is coated with a dense layer of carbohydrate groups, and that manages to evade immune system detection and to circulate for several months (before being removed by the same type of cell responsible for removing liposomes).

The first breakthrough came in 1987 when a glycolipid (the brain tissue-derived ganglioside GM1), was identified that, when incorporated within the lipid matrix, allowed liposomes to circulate for many hours in the blood stream (Allen and Chonn, 1987). A second glycolipid, phosphatidylinositol, was also found to impart long plasma residence times to liposomes and, since it was extracted from soybeans, not brain tissue, was believed to be a more pharmaceutically acceptable excipient (Gabizon et al., 1989).

A major advance in the surface-modified sub-branch was the development of polymer-coated liposomes (Allen et al. 1991). Polyethylene glycol (PEG) modification had been used for many years to prolong the half-lives of biological proteins (such as enzymes and growth factors) and to reduce their immunogenicity (*e.g.* Beauchamp et al., 1983). It was reported in the early 1990s that PEG-coated liposomes circulated for remarkably long times after intravenous administration. Half-lives on the order of 24 h were seen in mice and rats, and over 30 hours in dogs. The term "stealth" was applied to these liposomes because of their ability of evade interception by the immune system. The PEG hydrophilic polymers form dense "conformational clouds" to prevent other macromolecules from interaction with the

surface, even at low concentrations of the protecting polymer (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991; reviewed by Torchilin, 1998). The increased hydrophilicity of the liposomes after their coating with the amphipathic PEG5000 leads to a reduction in nonspecific uptake by the reticuloendothelial system.

Whereas the half-life of antimyosin immunoliposomes was 40 min, by coating with PEG, they increased their half-life to 1000 min after intravenous injection to rabbits (Torchilin et al., 1992).

Micelles, surfactants and small unilamellar vesicles

In a further embodiment, the liposome encapsulated DNA described herein, further comprise an initial step of micelle formation between cationic lipids and condensed plasmid or oligonucleotide DNA in ethanol solutions. Micelles are small amphiphilic colloidal particles formed by certain kinds of lipid molecules, detergents or surfactants under defined conditions of concentration, solvent and temperature. They are composed of a single lipid layer. Micelles can have their hydrophilic head groups assembled exposing their hydrophobic tails to the solvent (for example in 30-60% aqueous ethanol solution) or can reverse their structures exposing their polar heads toward the solvent such as by lowering the concentration of the ethanol to below 10% (reverse micelles). Micelle systems are in thermodynamic equilibrium with the solvent molecules and environment. This results in constant phase changes, especially upon contact with biological materials, such as upon introduction to cell culture, injection to animals, dilution, contact with proteins or other macromolecules. These changes result in rapid micelle disassembly or flocculation. This is in contrast to the much higher stability of liposome bilayers.

Single-chain surfactants are able to form micelles (see Table 1, below). These include the anionic (sodium dodecyl sulfate, cholate or oleate) or cationic (cetyl-trimethylammonium bromide, CTAB) surfactants. CTAB, CTAC, and DOIC micelles yielded larger solubility gaps (lower concentration of colloidally suspended DNA) than corresponding SUV particles containing neutral lipid and CTAB (1:1) (Lasic, 1997).

Table 1: Molecules able to form micelles

Molecule	Reference
CTAB, CTAC, DOIC	Lasic, 1997
Detergent/phospholipid micelles	Lusa et al.,1998
Dodecyl betaine (amphoteric surfactant)	de la Maza et al., 1998
Dodecylphosphocholine cholate	Lasic, 1997
Glycine-conjugated bile salt (anionic steroid detergent-like molecule)	Leonard and Cohen, 1998
Lipid-dodecyl maltoside micelles	Lambert et al., 1998
mixed micelles (Triton X-100 & phosphatidylcholine)	Lopez et al., 1998
Octylglucoside (non-ionic straight chain detergent)	Leonard and Cohen, 1998
Oleate	Lasic, 1997
PEG- dialkylphosphatidic acid (dihexadecylphosphatidyl (DHP)-PEG2000)	Tirosh et al., 1998
Phosphatidylcholine (neutral zwitterionic)	Schroeder et al., 1990
Polyethyleneglycol (MW 5000)-distearoyl phosphatidyl ethanolamine (PEG-DSPE)	Weissig et al., 1998
sodium dodecyl sulfate (anionic straight chain detergent)	Leonard and Cohen, 1998
Sodium taurofusidate (conjugated fungal bile salt analog)	Leonard and Cohen, 1998
Taurine- conjugated bile salts (anionic steroid detergent-like molecule)	Leonard and Cohen, 1998
Triton X-100 surfactant	Lasic, 1997

There is a critical detergent/phospholipid ratio at which lamellar-to-micellar transition occurs. For example, the vesicle-micelle transition was observed for dodecyl maltoside with large unilamellar liposomes. A striking feature of the solubilization process by dodecyl maltoside was the discovery of a new phase, consisting of a very viscous "gel-like" structure composed of long filamentous thread-like micelles, over 1 to 2 microns in length.

A long circulating complex needs to be slightly anionic. Therefore the liposomes used for the conversion of the micelles into liposomes contain bipolar lipids (PC, PE) and 1-30% negatively charged lipids (DPPG). The cationic lipids which are toxic, are hidden in the inner liposome membrane bilayer. Those reaching the solid tumor will exert their toxic effects causing apoptosis. Apoptosis will be caused by the delivery of the toxic drug or anti-neoplastic gene or oligonucleotide to the cancer cell but also by the nuclear localization of the cationic lipids (along with plasmid DNA) to the nucleus. Indeed, a number of studies suggest that plasmid DNA is imported to nuclei; its translocation docks cationic lipid molecules electrostatically attached to the DNA. These cationic lipid molecules exert their toxicity by interfering with the nucleosome and domain structure of the chromatin causing local destabilization. This disturbance or aberrant chromatin reorganization

could be exerted at the level of the nuclear matrix where plasmid DNA is attached for transcription, autonomous replication, or integration via recombination.

Surfactants have found wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, *in:* Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, 1988, p. 285).

Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general, their HLB values range from 2 to about 18, depending on their structure. Nonionic surfactants include, nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers, such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated, block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class. If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

Classical micelles may not be effective as gene transfer vehicles, but important intermediates in the formation of liposome complexes encapsulating drugs or nucleic acids. The stability of single chain surfactants-DNA-colloidal systems is lower than SUV particles containing neutral lipid and CTAB (1:1). However,

second generation micelles are able to target tumors *in vivo*. Weissig and co-workers (1998) used the soybean trypsin inhibitor (STI) as a model protein to target tumors. STI was modified with a hydrophobic residue of N-glutaryl-phosphatidyl-ethanolamine (NGPE) and incorporated into both polyethyleneglycol (MW 5000)-distearoyl phosphatidyl ethanolamine (PEG-DSPE) micelles (< 20 nm) and PEG-DSPE-modified long-circulating liposomes (ca. 100 nm). As determined from the protein label by using ^{111}In attached to soybean trypsin inhibitor via protein-attached diethylene triamine pentaacetic acid, DTPA, PEG-lipid micelles accumulated better than the same protein anchored in long-circulating PEG-liposomes in subcutaneously established Lewis lung carcinoma in mice after tail vein injection.

Loading a liposomal dispersion with an amphiphilic drug may cause a phase transformation into a micellar solution. The transition from high ratios of phospholipid to drug (from 2:1 to 1:1 downwards) were accompanied by the conversion of liposomal dispersions of milky-white appearance (particle size 200 nm) to nearly transparent micelles (particle size below 25 nm). See, Schutze and Muller-Goymann (1998).

Fusogenic peptides

In a further embodiment, the liposome encapsulated DNA described herein further comprises an effective amount of a fusogenic peptide. Fusogenic peptides belong to a class of helical amphipathic peptides characterized by a hydrophobicity gradient along the long helical axis. This hydrophobicity gradient causes the tilted insertion of the peptides in membranes, thus destabilizing the lipid core and, thereby, enhancing membrane fusion (Decout et al., 1999).

Hemagglutinin (HA) is a homotrimeric surface glycoprotein of the influenza virus. In infection, it induces membrane fusion between viral and endosomal membranes at low pH. Each monomer consists of the receptor-binding HA1 domain and the membrane-interacting HA2 domain. The NH_2-terminal region of the HA2 domain (amino acids 1 to 127), the so-called "fusion peptide," inserts into the target membrane and plays a crucial role in triggering fusion between the viral and endosomal membranes. Based on the substitution of eight amino acids in region 5-14 with cysteines and spin-labeling electron paramagnetic resonance, it was

concluded that the peptide forms an alpha-helix tilted approximately 25 degrees from the horizontal plane of the membrane with a maximum depth of 15 Å from the phosphate group (Macosko et al., 1997). Use of fusogenic peptides from influenza virus hemagglutinin HA-2 enhanced greatly the efficiency of transferrin-polylysine-DNA complex uptake by cells. The peptide was linked to polylysine and the complex was delivered by the transferrin receptor-mediated endocytosis (reviewed by Boulikas, 1998a). This peptide has the sequence: GLFEAIAGFI ENGWEGMIDG GGYC (SEQ ID NO:9) and is able to induce the release of the fluorescent dye calcein from liposomes prepared with egg yolk phosphatidylcholine, which was higher at acidic pH. This peptide was also able to increase up to 10-fold the anti-HIV potency of antisense oligonucleotides, at a concentration of 0.1-1 mM, using CEM-SS lymphocytes in culture. This peptide changes conformation at the slightly more acidic environment of the endosome, destabilizing and breaking the endosomal membrane (reviewed by Boulikas, 1998a).

The presence of negatively charged lipids in the membrane is important for the manifestation of the fusogenic properties of some peptides, but not of others. Whereas the fusogenic action of a peptide, representing a putative fusion domain of fertilin, a sperm surface protein involved in sperm-egg fusion, was dependent upon the presence of negatively charged lipids, that of the HIV2 peptide was not (Martin and Ruysschaert, 1997).

For example, to analyze the two domains on the fusogenic peptides of influenza virus hemagglutinin HA, HA-chimeras were designed in which the cytoplasmic tail and/or transmembrane domain of HA was replaced with the corresponding domains of the fusogenic glycoprotein F of Sendai virus. Constructs of HA were made in which the cytoplasmic tail was replaced by peptides of human neurofibromin type 1 (NF1) (residues 1441 to 1518) or c-Raf-1, (residues 51 to 131) and were expressed in CV-1 cells by using the vaccinia virus-T7 polymerase transient-expression system. Membrane fusion between CV-1 cells and bound human erythrocytes (RBCs) mediated by parental or chimeric HA proteins showed that, after the pH was lowered, a flow of the aqueous fluorophore calcein from preloaded RBCs into the cytoplasm of the protein-expressing CV-1 cells took place.

This indicated that membrane fusion involves both leaflets of the lipid bilayers and leads to formation of an aqueous fusion pore (Schroth-Diaz et al., 1998).

A remarkable discovery was that the TAT protein of HIV is able to cross cell membranes (Green and Loewenstein, 1998) and that a 36-amino acid domain of TAT, when chemically cross-linked to heterologous proteins, conferred the ability to transduce into cells. The 11-amino acid fusogenic peptide of TAT (YGRKKRRQRRR (SEQ ID NO:10)) is a nucleolar localization signal (see Boulikas, 1998b).

Another protein of HIV, the glycoprotein gp41, contains fusogenic peptides. Linear peptides derived from the membrane proximal region of the gp41 ectodomain have potential applications as anti-HIV agents and inhibit infectivity by adopting a helical conformation (Judice et al., 1997). The 23 amino acid residue, N-terminal peptide of HIV-1 gp41 has the capacity to destabilize negatively charged large unilamellar vesicles. In the absence of cations, the main structure was a pore-forming alpha-helix, whereas in the presence of Ca^{2+} the conformation switched to a fusogenic, predominantly extended beta-type structure. The fusion activity of HIV(ala) (bearing the R22→A substitution) was reduced by 70%, whereas fusogenicity was completely abolished when a second substitution (V2→E) was included, arguing that it is not an alpha-helical but an extended structure adopted by the HIV-1 fusion peptide that actively destabilizes cholesterol-containing, electrically neutral membranes (Pereira et al., 1997).

The prion protein (PrP) is a glycoprotein of unknown function normally found at the surface of neurons and of glial cells. It is involved in diseases such as bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease in humans, where PrP is converted into an altered form (termed PrPSc). According to computer modeling calculations, the 120 to 133 and 118 to 135 domains of PrP are tilted lipid-associating peptides inserting in a oblique way into a lipid bilayer and able to interact with liposomes to induce leakage of encapsulated calcein (Pillot et al., 1997b).

The C-terminal fragments of the Alzheimer amyloid peptide (amino acids 29-40 and 29-42) have properties related to those of the fusion peptides of viral proteins inducing fusion of liposomes *in vitro*. These properties could mediate a direct

interaction of the amyloid peptide with cell membranes and account for part of the cytotoxicity of the amyloid peptide. In view of the epidemiologic and biochemical linkages between the pathology of Alzheimer's disease and apolipoprotein E (apoE) polymorphism, examination of the potential interaction between the three common apoE isoforms and the C-terminal fragments of the amyloid peptide showed that only apoE2 and apoE3, not apoE4, are potent inhibitors of the amyloid peptide fusogenic and aggregational properties. The protective effect of apoE against the formation of amyloid aggregates was thought to be mediated by the formation of stable apoE/amyloid peptide complexes (Pillot et al., 1997a; Lins et al., 1999).

The fusogenic properties of an amphipathic net-negative peptide (WAE 11), consisting of 11 amino acid residues were strongly promoted when the peptide was anchored to a liposomal membrane. The fusion activity of the peptide appeared to be independent of pH and membrane merging, and the target membranes required a positive charge that was provided by incorporating lysine-coupled phosphatidylethanolamine (PE-K). Whereas the coupled peptide could cause vesicle aggregation via nonspecific electrostatic interaction with PE-K, the free peptide failed to induce aggregation of PE-K vesicles (Pecheur et al., 1997).

A number of studies suggest that stabilization of an alpha-helical secondary structure of the peptide after insertion in lipid bilayers in membranes of cells or liposomes is responsible for the membrane fusion properties of peptides. Zn^{2+}, enhances the fusogenic activity of peptides because it stabilizes the alpha-helical structure. For example, the HEXXH (SEQ ID NO:11) domain of the salivary antimicrobial peptide, located in the C-terminal functional domain of histatin-5, a recognized zinc-binding motif is in a helicoidal conformation (Martin et al., 1999; Melino et al., 1999; Curtain et al., 1999).

Fusion peptides have been formulated with DNA plasmids to create peptide-based gene delivery systems. A combination of the YKAKnWK (SEQ ID NO:12) peptide, used to condense plasmids into 40 to 200 nm nanoparticles, with the GLFEALLELLESLWELLLEA (SEQ ID NO:13) amphipathic peptide, that is a pH-sensitive lytic agent designed to facilitate release of the plasmid from endosomes enhanced expression systems containing the beta-galactosidase reporter gene (Duguid et al., 1998). See Table 2, below.

Table 2. Fusogenic peptides

Fusogenic peptide	Source Protein	Properties	Reference
GLFEAIAGFIENGWEG MIDGGGYC (SEQ ID NO:9)	Influenza virus hemagglutinin HA-2	Endowed with membrane fusion properties	Bongartz et al., 1994
YGRKKRRQRRR (SEQ ID NO:5)	TAT of HIV	Endowed with membrane fusion properties	Green and Loewenstein, 1988
23-residue fusogenic N-terminal peptide	HIV-1 trans-membrane glycoprotein gp41	Was able to insert as an alpha-helix into neutral phospholipid bilayers	Curtain et al., 1999
70 residue peptide (SV-117)	Fusion peptide and N-terminal heptad repeat of Sendai virus	Induced lipid mixing of egg phosphatidylcholine-phosphatidyiglycerol (PC/PG) large unilamellar vesicles (LUVs)	Ghosh and Shai, 1999
23 hydrophobic amino acids in the amino-terminal region	S protein of hepatitis B virus (HBV)	A high degree of similarity with known fusogenic peptides from other viruses.	Rodriguez-Crespo et al., 1994
MSGTFGGILAGLIGLL (SEQ ID NO:6)	N-terminal region of the S protein of duck hepatitis B Virus (DHBV)	Was inserted into the hydrophobic core of the lipid bilayer and induced leakage of internal aqueous contents from both neutral and negatively charged liposomes	Rodriguez-Crespo et al., 1999
MSPSSLLGLLAGLQVV (SEQ ID NO:14)	S protein of woodchuck hepatitis B virus (WHV)	Was inserted into the hydrophobic core of the lipid bilayer and induced leakage of internal aqueous contents from both neutral and negatively charged liposomes	Rodriguez-Crespo et al., 1999
N-terminus of Nef	Nef protein of human immunodeficienc y type 1 (HIV-1)	Membrane-perturbing and fusogenic activities in artificial membranes; causes cell killing in E. coli and yeast	Macreadie et al., 1997
Amino-terminal sequence F1 polypeptide	F1 polypeptide of measles virus (MV)	Can be used as a carrier system for CTL epitopes	Partidos et al., 1996
19-27 amino acid segment	Glycoprotein gp51 of bovine leukemia virus	Adopts an amphiphilic structure and plays a key role in the fusion events induced by bovine leukemia virus	Voneche et al., 1992
120 to 133 and 118 to 135 domains	Prion protein	Tilted lipid-associating peptide; interact with liposomes to induce leakage of encapsulated calcein	Pillot et al., 1997b
29-42-residue fragment	Alzheimer's beta-amyloid peptide	Endowed with capacities resembling those of the tilted fragment of viral fusion proteins	Lins et al., 1999
Non-aggregated amyloid beta-peptide (1-40)	Alzheimer's beta-amyloid peptide	Induces apoptotic neuronal cell death	Pillot et al., 1999

Fusogenic peptide	Source Protein	Properties	Reference
LCAT 56-68 helical segment	Lecithin cholesterol acyltransferase (LCAT)	Forms stable beta-sheets in lipids	Peelman et al., 1999; Decout et al., 1999
Peptide sequence B18	Membrane-associated sea urchin sperm protein binding	Triggers fusion between lipid vesicles; a histidine-rich motif for binding zinc is required for the fusogenic function	Ulrich et al., 1999
53-70 (C-terminal helix)	Apolipoprotein (apo) AII	Induces fusion of unilamellar lipid vesicles and displaces apo AI from HDL and r-HDL	Lambert et al., 1998
Residues 90-111	PH-30 alpha (a protein functioning in sperm-egg fusion)	Membrane-fusogenic activity to acidic phospholipid bilayers	Niidome et al., 1997
Casein signal peptides	Alpha s2- and beta-casein	Interact with dimyristoylphosphatidyl-glycerol and -choline liposomes; show both lytic and fusogenic activities	Creuzenet et al., 1997
Pardaxin	Amphipathic polypeptide, purified from the gland secretion of the Red Sea Moses sole flatfish Pardachirus marmoratus	Forms voltage-gated, cation-selective pores; mediated the aggregation of liposomes composed of phosphatidylserine but not of phosphatidylcholine	Lelkes and Lazarovici, 1988
Histatin-5	Salivary antimicrobial peptide	Aggregates and fuses negatively charged small unilamellar vesicles in the presence of Zn2+	Melino et al., 1999
Gramicidin (linear hydrophobic polypeptide)	Antibiotic	Induces aggregation and fusion of vesicles	Massari and Colonna, 1986; Tournois et al., 1990
Amphipathic negatively charged peptide consisting of 11 residues (WAE)	Synthetic	Forms an alpha-helix inserted and anchored into the membrane (favored at 37oC) oriented almost parallel to the lipid acyl chains; promotes fusion of large unilamellar liposomes (LUV)	Martin et al., 1999
A polymer of polylysine (average 190) partially substituted with histidyl residues	Synthetic	Histidyl residues become cationic upon protonation of the imidazole groups at pH below 6.0.; disrupt endosomal membranes	Midoux and Monsigny, 1999
GLFEALLELLESLWELL LEA (SEQ ID NO:4)	Synthetic	Amphipathic peptide; a pH-sensitive lytic agent to facilitate release of the plasmid from endosomes	Duguid et al., 1998

Fusogenic peptide	Source Protein	Properties	Reference
$(LKKL)_4$ (SEQ ID NO:15)	Synthetic	Amphiphilic fusogenic peptide, able to interact with four molecules of DMPC	Gupta and Kothekar, 1997
Ac-$(Leu\text{-}Ala\text{-}Arg\text{-}Leu)_3$-$NHCH_3$ (SEQ ID NO:16)	Synthetic; basic amphipathic peptides	Caused a leakage of contents from small unilamellar vesicles composed of egg yolk phosphatidylcholine and egg yolk phosphatidic acid (3:1)	Suenaga et al., 1989; Lee et al., 1992
Amphiphilic anionic peptides E5 and E5L	Synthetic	Can mimic the fusogenic activity of influenza hemagglutinin (HA)	Murata et al., 1991
30-amino acid peptide with the major repeat unit Glu-Ala-Leu-Ala $(GALA)_7$ (SEQ ID NO:17)	Synthetic; designed to mimic the behavior of the fusogenic sequences of viral fusion proteins	Becomes an amphipathic alpha-helix as the pH is lowered to 5.0 ; fusion of phosphatidylcholine small unilamellar vesicles induced by GALA requires a peptide length greater than 16 amino acids	Parente et al., 1988
Poly Glu-Aib-Leu-Aib (SEQ ID NO:18) Aib represents 2-aminoisobutyric acid	Synthetic	Amphiphilic structure upon the formation of alpha-helix; caused fusion of EYPC liposomes and dipalmitoylphosphatidylcholine liposomes more strongly with decreasing pH	Kono et al., 1993

Fusogenic lipids

DOPE is a fusogenic lipid; elastase cleavage of N-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE (SEQ ID NO:19) converted this derivative to DOPE (overall positive charge) to deliver an encapsulated fluorescent probe, calcein, into the cell cytoplasm (Pak et al., 1999). An oligodeoxynucleic sequence of 30 bases complementary to a region of beta-endorphin mRNA elicited a concentration-dependent inhibition of beta-endorphin production in cell culture after it was encapsulated within small unilamellar vesicles (50 nm) containing dipalmitoyl-DL-alpha-phosphatidyl-L-serine endowed with fusogenic properties (Fresta et al., 1998).

Nuclear localization signals (NLS)

In a further embodiment, the liposome encapsulated plasmid or oligonucleotide DNA described herein further comprise an effective amount of nuclear localization signal (NLS) peptides. Trafficking of nuclear proteins from the site of their synthesis in the cytoplasm to the sites of function in the nucleus through

pore complexes is mediated by NLSs on proteins to be imported into nuclei (Tables 3-10, below). Protein translocation from the cytoplasm to the nucleoplasm involves: (i) the formation of a complex of karyopherin α with NLS-protein; (ii) subsequent binding of karyopherin β; (iii) binding of the complex to FXFG peptide repeats on nucleoporins; (iv) docking of Ran-GDP to nucleoporin and to karyopherin heterodimer by p10; (v) a number of association-dissociation reactions on nucleoporins that dock the import substrate toward the nucleoplasmic side with a concomitant GDP-GTP exchange reaction transforming Ran-GDP into Ran-GTP and catalyzed by karyopherin α; and (vi) dissociation from karyopherin β and release of the karyopherin α/NLS-protein by Ran-GTP to the nucleoplasm.

Karyophilic and acidic clusters were found in most non-membrane serine/threonine protein kinases whose primary structure has been examined (Table 6). These karyophilic clusters might mediate the anchoring of the kinase molecules to transporter proteins for their regulated nuclear import and might constitute the nuclear localization signals. In contrast to protein transcription factors that are exclusively nuclear possessing strong karyophilic peptides composed of at least four arginines, (R), and lysines, (K), within an hexapeptide flanked by proline and glycine helix-breakers, protein kinases often contain one histidine and three K+R residues (Boulikas, 1996). This was proposed to specify a weak NLS structure resulting in the nuclear import of a fraction of the total cytoplasmic kinase molecules, as well as in their weak retention in the different ionic strength nuclear environment. Putative NLS peptides in protein kinases may also contain hydrophobic or bulky aromatic amino acids proposed to further diminish their capacity to act as strong NLS.

Most mammalian proteins that participate in DNA repair pathways seem to possess strong karyophilic clusters containing at least four R+K over a stretch of six amino acids (Table 7).

Rules to predict nuclear localization of an unknown protein

Several simple rules have been proposed for the prediction of the nuclear localization of a protein of an unknown function from its amino acid sequence:

(i) An NLS is defined as four arginines (R) plus lysines (K) within an hexapeptide; the presence of one or more histidines (H) in the tetrad of the karyophilic hexapeptide, often found in protein kinases that have a cytoplasmic and a nuclear function, may specify a weak NLS whose function might be regulated by phosphorylation or may specify proteins that function in both the cytoplasm and the nucleus (Boulikas, 1996);

(ii) The K/R clusters are flanked by the α-helix breakers G and P thus placing the NLS at a helix-turn-helix or end of a α-helix. Negatively-charged amino acids (D, E) are often found at the flank of the NLS and on some occasions may interrupt the positively-charged NLS cluster;

(iii) Bulky amino acids (W, F, Y) are not present within the NLS hexapeptide;

(iv) NLS signals may not be flanked by long stretches of hydrophobic amino acids (*e.g.* five); a mixture of charged and hydrophobic amino acids serves as a mitochondrial targeting signal;

(v) The higher the number of NLSs, the more readily a molecule is imported to the nucleus (Dworetzky et al., 1988). Even small proteins, for example histones (10-22 kDa), need to be actively imported to increase their import rates compared with the slow rate of diffusion of small molecules through pores;

(vi) Signal peptides are stronger determinants than NLSs for protein trafficking. Signal peptides direct proteins to the lumen of the endoplasmic reticulum for their secretion or insertion into cellular membranes (presence of transmembrane domains) (Boulikas, 1994);

(vii) Signals for the mitochondrial import of proteins (a mixture of hydrophobic and karyophilic amino acids) may antagonize nuclear import signals and proteins possessing both type of signals may be translocated to both mitochondria and nuclei;

(viii) Strong association of a protein with large cytoplasmic structures (membrane proteins, intermediate filaments) make such proteins unavailable for import even though they posses NLS-like peptides (Boulikas, 1994);

(ix). Transcription factors and other nuclear proteins posses a great different number of putative NLS stretches. Of the sixteen possible forms of putative NLS

structures the most abundant types are the θθxθθ, θθθxθ, θθθθ, and θθxθxθ, where θ is R or K, together accounting for about 70% of all karyophilic clusters on transcription factors (Boulikas, 1994);

(x) A small number of nuclear proteins seem to be void of a typical karyophilic NLS. Either non karyophilic peptides function for their nuclear import, as such molecules possess bipartite NLSs, or these NLS-less proteins depend absolutely for import on their strong complexation in the cytoplasm with a nuclear protein partner able to be imported (Boulikas, 1994). This mechanism may ensure a certain stoichiometric ratio of the two molecules in the nucleus, and might be of physiological significance; and

(xi) A number of proteins may be imported via other mechanisms not dependent on classical NLS.

A number of processes have been found to be regulated by nuclear import including nuclear translocation of the transcription factors NF-κB, rNFIL-6, ISGF3, SRF, c-Fos, GR as well as human cyclins A and B1, casein kinase II, cAMP-dependent protein kinase II, protein kinase C, ERK1 and ERK2. Failure of cells to import specific proteins into nuclei can lead to carcinogenesis. For example, BRCA1 is mainly localized in the cytoplasm in breast and ovarian cancer cells, whereas in normal cells the protein is nuclear. mRNA is exported through the same route as a complex with nuclear proteins possessing nuclear export signals (NES). The majority of proteins with NES are RNA-binding proteins that bind to and escort RNAs to the cytoplasm. However, other proteins with NES function in the export of proteins; CRM1, that binds to the NES sequence on other proteins and interacts with the nuclear pore complex, is an essential mediator of the NES-dependent nuclear export of proteins in eukaryotic cells. Nuclear localization and export signals (NLS and NES) are found on a number of important molecules, including p53, v-Rel, the transcription factor NF-ATc, the c-Abl nonreceptor tyrosine kinase, and the fragile X syndrome mental retardation gene product. The deregulation of their normal import/export trafficking has important implications for human disease. Both nuclear import and export processes can be manipulated by conjugation of proteins with NLS or NES peptides. During gene therapy, the foreign DNA needs to enter nuclei for its transcription. A pathway is proposed involving the complexation of

plasmids and oligonucleotides with nascent nuclear proteins possessing NLSs as a prerequisite for their nuclear import. Covalent linkage of NLS peptides to oligonucleotides and plasmids or formation of complexes of plasmids with proteins possessing multiple NLS peptides was proposed (Boulikas, 1998b) to increase their import rates and the efficiency of gene expression. Cancer cells were predicted to import more efficiently foreign DNA into nuclei, compared with terminally differentiated cells because of their increased rates of proliferation and protein import.

Antineoplastic drugs

In a further embodiment, the liposome encapsulated plasmid or oligonucleotide DNA described herein, further comprises its use for reducing tumor size or restricting its growth with combination with encapsulated or free antineoplastic agents. Antineoplastic agents preferably are: **(i)** alkylating agents having the bis-(2-chloroethyl)-amine group such as chlormethine, chlorambucile, melphalan, uramustine, mannomustine, extramustinephosphat, mechlorethaminoxide, cyclophosphamide, ifosfamide, or trifosfamide; **(ii)** alkylating agents having a substituted aziridine group, for example tretamine, thiotepa, triaziquone, or mitomycine; **(iii)** alkylating agents of the methanesulfonic ester type such as busulfane; **(iv)** alkylating N-alkyl-N-nitrosourea derivatives, for example carmustine, lomustine, semustine, or streptozotocine; **(v)** alkylating agents of the mitobronitole, dacarbazine, or procarbazine type; **(vi)** complexing agents such as cis-platin; **(vii)** antimetabolites of the folic acid type, for example methotrexate; **(viii)** purine derivatives such as mercaptopurine, thioguanine, azathioprine, tiamiprine, vidarabine, or puromycine and purine nucleoside phosphorylase inhibitors; **(ix)** pyrimidine derivatives, for example fluorouracil, floxuridine, tegafur, cytarabine, idoxuridine, flucytosine; **(x)** antibiotics such as dactinomycin, daunorubicin, doxorubicin, mithramycin, bleomycin or etoposide; **(xi)** vinca alkaloids; **(xii)** inhibitors of proteins overexpressed in cancer cells such as telomerase inhibitors, glutathione inhibitors, proteasome inhibitors; **(xiii)** modulators or inhibitors of signal transduction pathways such as phosphatase inhibitors, protein kinase C inhibitors, casein kinase inhibitors, insulin-like growth factor-1 receptor inhibitor, ras

inhibitors, ras-GAP inhibitor, protein tyrosine phosphatase inhibitors; (**xiv**) tumor angiogenesis inhibitors such as angiostatin, oncostatin, endostatin, thalidomide; (**xv**) modulators of the immune response and cytokines such as interferons, interleukins, TNF-alpha; (**xvi**) modulators of the extracellular matrix such as matrix metalloproteinase inhibitors, stromelysin inhibitors, plasminogen activator inhibitor; (**xvii**) hormone modulators for hormone-dependent cancers (breast cancer, prostate cancer) such as antiandrogen, estrogens; (**xviii**) apoptosis regulators; (**xix**) bFGF inhibitor; (**xx**) multiple drug resistance gene inhibitor; (**xxi**) monoclonal antibodies or antibody fragments against antigenes overexpressed in cancer cells (anti-Her2/neu for breast cancer); (**xxii**) anticancer genes whose expression will cause apoptosis, arrest the cell cycle, induce an immune response against cancer cells, inhibit tumor angiogenesis *i.e.* formation of blood vessels, tumor suppressor genes (p53, RB, BRCA1, E1A, bcl-2, MDR-1, p21, p16, bax, bcl-xs, E2F, IGF-I VEGF, angiostatin, oncostatin, endostatin, GM-CSF, IL-12, IL-2, IL-4, IL-7, IFN-γ, and TNF-α); and (**xxiii**) antisense oligonucleotides (antisense c-fos, c-myc, K-ras). Optionally these drugs are administered in combination with chlormethamine, prednisolone, prednisone, or procarbazine or combined with radiation therapy. Future new anticancer drugs added to the arsenal are expected to be ribozymes, triplex-forming oligonucleotides, gene inactivating oligonucleotides, a number of new genes directed against genes that control the cell proliferation or signaling pathways, and compounds that block signal transduction.

Anti-cancer drugs include: acivicin, aclarubicin, acodazole hydrochloride, acronine, adozelesin, adriamycin, aldesleukin, altretamine, ambomycin, ametantrone acetate, aminoglutethimide, amsacrine, anastrozole, anthramycin, asparaginase, asperlin, azacitidine, azetepa, azotomycin, batimastat, benzodepa, bicalutamide, bisantrene hydrochloride, bisnafide dimesylate, bizelesin, bleomycin sulfate, brequinar sodium, bropirimine, busulfan, cactinomycin, calusterone, caracemide, carbetimer, carboplatin, carmustine, carubicin hydrochloride, carzelesin, cedefingol, chlorambucil, cirolemycin, cisplatin, cladribine, crisnatol mesylate, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin hydrochloride, decitabine, dexormaplatin, dezaguanine, dezaguanine mesylate, diaziquone, docetaxel, doxorubicin, doxorubicin hydrochloride, droloxifene,

droloxifene citrate, dromostanolone propionate, duazomycin, edatrexate, eflornithine hydrochloride, elsamitrucin, enloplatin, enpromate, epipropidine, epirubicin hydrochloride, erbulozole, esorubicin hydrochloride, estramustine, estramustine phosphate sodium, etanidazole, etoposide, etoposide phosphate, etoprine, fadrozole hydrochloride, fazarabine, fenretinide, floxuridine, fludarabine phosphate, fluorouracil, flurocitabine, fosquidone, fostriecin sodium, gemcitabine, gemcitabine hydrochloride, hydroxyurea, idarubicin hydrochloride, ifosfamide, ilmofosine, interferon alfa-2a, interferon α-2b, interferon α-n1, interferon α-n3, interferon β-i a, interferon γ-i b, iproplatin, irinotecan hydrochloride, lanreotide acetate, letrozole, leuprolide acetate, liarozole hydrochloride, lometrexol sodium, lomustine, losoxantrone hydrochloride, masoprocol, maytansine, mechlorethamine hydrochloride, megestrol acetate, melengestrol acetate, melphalan, menogaril, mercaptopurine, methotrexate, methotrexate sodium, metoprine, meturedepa, mitindomide, mitocarcin, mitocromin, mitogillin, mitomalcin, mitomycin, mitosper, mitotane, mitoxantrone hydrochloride, mycophenolic acid, nocodazole, nogalamycin, ormaplatin, oxisuran, paclitaxel, pegaspargase, peliomycin, pentamustine, peplomycin sulfate, perfosfamide, pipobroman, piposulfan, piroxantrone hydrochloride, plicamycin, plomestane, porfimer sodium, porfiromycin, prednimustine, prednisone, procarbazine hydrochloride, puromycin, puromycin hydrochloride, pyrazofurin, riboprine, rogletimide, safingol, safingol hydrochloride, semustine, simtrazene, sparfosate sodium, sparsomycin, spirogermanium hydrochloride, spiromustine, spiroplatin, streptonigrin, streptozocin, sulofenur, talisomycin, taxol, tecogalan sodium, tegafur, teloxantrone hydrochloride, temoporfin, teniposide, teroxirone, testolactone, thiamiprine, thioguanine, thiotepa, tiazofurin, tirapazamine, topotecan hydrochloride, toremifene citrate, trestolone acetate, triciribine phosphate, trimetrexate, trimetrexate glucuronate, triptorelin, tubulozole hydrochloride, uracil mustard, uredepa, vapreotide, verteporfin, vinblastine sulfate, vincristine sulfate, vindesine, vindesine sulfate, vinepidine sulfate, vinglycinate sulfate, vinleurosine sulfate, vinorelbine tartrate, vinrosidine sulfate, vinzolidine sulfate, vorozole, zeniplatin, zinostatin, zorubicin hydrochloride.

Other anti-cancer drugs include: 20-epi-1,25 dihydroxyvitamin D3, 5-ethynyluracil, abiraterone, aclarubicin, acylfulvene, adecypenol, adozelesin,

aldesleukin, ALL-TK antagonists, altretamine, ambamustine, amidox, amifostine, aminolevulinic acid, amrubicin, amsacrine, anagrelide, anastrozole, andrographolide, angiogenesis inhibitors, antagonist D, antagonist G, antarelix, anti-dorsalizing morphogenetic protein-1, antiandrogen, antiestrogen, antineoplaston, antisense oligonucleotides, aphidicolin glycinate, apoptosis gene modulators, apoptosis regulators, apurinic acid, ara-CDP-DL-PTBA, arginine deaminase, asulacrine, atamestane, atrimustine, axinastatin 1, axinastatin 2, axinastatin 3, azasetron, azatoxin, azatyrosine, baccatin III derivatives, balanol, batimastat, BCR/ABL antagonists, benzochlorins, benzoylstaurosporine, beta lactam derivatives, beta-alethine, betaclamycin B, betulinic acid, bFGF inhibitor, bicalutamide, bisantrene, bisaziridinylspermine, bisnafide, bistratene A, bizelesin, breflate, bropirimine, budotitane, buthionine sulfoximine, calcipotriol, calphostin C, camptothecin derivatives, canarypox IL-2, capecitabine, carboxamide-amino-triazole, carboxyamidotriazole, CaRest M3, CARN 700, cartilage derived inhibitor, carzelesin, casein kinase inhibitors (ICOS), castanospermine, cecropin B, cetrorelix, chlorlns, chloroquinoxaline sulfonamide, cicaprost, cis-porphyrin, cladribine, clomifene analogues, clotrimazole, collismycin A, collismycin B, combretastatin A4, combretastatin analogue, conagenin, crambescidin 816, crisnatol, cryptophycin 8, cryptophycin A derivatives, curacin A, cyclopentanthraquinones, cycloplatam, cypemycin, cytarabine ocfosfate, cytolytic factor, cytostatin, dacliximab, decitabine, dehydrodidemnin B, deslorelin, dexifosfamide, dexrazoxane, dexverapamil, diaziquone, didemnin B, didox, diethylnorspermine, dihydro-5-azacytidine, dihydrotaxol, 9-dioxamycin, diphenyl spiromustine, docosanol, dolasetron, doxifluridine, droloxifene, dronabinol, duocarmycin SA, ebselen, ecomustine, edelfosine, edrecolomab, eflornithine, elemene, emitefur, epirubicin, epristeride, estramustine analogue, estrogen agonists, estrogen antagonists, etanidazole, etoposide phosphate, exemestane, fadrozole, fazarabine, fenretinide, filgrastim, finasteride, flavopiridol, flezelastine, fluasterone, fludarabine, fluorodaunorunicin hydrochloride, forfenimex, formestane, fostriecin, fotemustine, gadolinium gallium nitrate texaphyrin, galocitabine, ganirelix, gelatinase inhibitors, gemcitabine, glutathione inhibitors, hepsulfam, heregulin, hexamethylene bisacetamide, hypericin, ibandronic acid, idarubicin, idoxifene, idramantone, ilmofosine, ilomastat,

imidazoacridones, imiquimod, immunostimulant peptides, insulin-like growth factor-1 receptor inhibitor, interferon agonists, interferons, interleukins, iobenguane, iododoxorubicin, ipomeanol, 4-, irinotecan, iroplact, irsogladine, isobengazole, isohomohalicondrin B, itasetron, jasplakinolide, kahalalide F, lamellarin-N triacetate, lanreotide, leinamycin, lenograstim, lentinan sulfate, leptolstatin, letrozole, leukemia inhibiting factor, leukocyte alpha interferon, leuprolide+estrogen+progesterone, leuprorelin, levamisole, liarozole, linear polyamine analogue, lipophilic disaccharide peptide, lipophilic platinum compounds, lissoclinamide 7, lobaplatin, lombricine, lometrexol, lonidamine, losoxantrone, lovastatin, loxoribine, lurtotecan, lutetium texaphyrin, lysofylline, lytic peptides, maitansine, mannostatin A, marimastat, masoprocol, maspin, matrilysin inhibitors, matrix metalloproteinase inhibitors, menogaril, merbarone, meterelin, methioninase, metoclopramide, MIF inhibitor, mifepristone, miltefosine, mirimostim, mismatched double stranded RNA, mitoguazone, mitolactol, mitomycin analogues, mitonafide, mitotoxin fibroblast growth factor-saporin, mitoxantrone, mofarotene, molgramostim, monoclonal antibody, human chorionic gonadotrophin, monophosphoryl lipid A+myobacterium cell wall sk, mopidamol, multiple drug resistance gene inhibitor, multiple tumor suppressor 1-based therapy, mustard anticancer agent, mycaperoxide B, mycobacterial cell wall extract, myriaporone, N-acetyldinaline, N-substituted benzamides, nafarelin, nagrestip, naloxone +pentazocine, napavin, naphterpin, nartograstim, nedaplatin, nemorubicin, neridronic acid, neutral endopeptidase, nilutamide, nisamycin, nitric oxide modulators, nitroxide antioxidant, nitrullyn, O6-benzylguanine, octreotide, okicenone, oligonucleotides, onapristone, ondansetron, ondansetron, oracin, oral cytokine inducer, ormaplatin, osaterone, oxaliplatin, oxaunomycin, paclitaxel analogues, paclitaxel derivatives, palauamine, palmitoylrhizoxin, pamidronic acid, panaxytriol, panomifene, parabactin, pazelliptine, pegaspargase, peldesine, pentosan polysulfate sodium, pentostatin, pentrozole, perflubron, perfosfamide, perillyl alcohol, phenazinomycin, phenylacetate, phosphatase inhibitors, picibanil, pilocarpine hydrochloride, pirarubicin, piritrexim, placetin A, placetin B, plasminogen activator inhibitor, platinum complex, platinum compounds, platinum-triamine complex, porfimer sodium, porfiromycin, propyl bis-acridone,

prostaglandin J2, proteasome inhibitors, protein A-based immune modulator, protein kinase C inhibitor, protein kinase C inhibitors, microalgal., protein tyrosine phosphatase inhibitors, purine nucleoside phosphorylase inhibitors, purpurins, pyrazoloacridine, pyridoxylated hemoglobin polyoxyethylene conjugate, raf antagonists, raltitrexed, ramosetron, ras farnesyl protein transferase inhibitors, ras inhibitors, ras-GAP inhibitor, retelliptine demethylated, rhenium Re 186 etidronate, rhizoxin, ribozymes, RII retinamide, rogletimide, rohitukine, romurtide, roquinimex, rubiginone B1, ruboxyl, safingol, saintopin, SarCNU, sarcophytol A, sargramostim, Sdi 1 mimetics, semustine, senescence derived inhibitor 1, sense oligonucleotides, signal transduction inhibitors, signal transduction modulators, single chain antigen binding protein, sizofiran, sobuzoxane, sodium borocaptate, sodium phenylacetate, solverol, somatomedin binding protein, sonermin, sparfosic acid, spicamycin D, spiromustine, splenopentin, spongistatin 1, squalamine, stem cell inhibitor, stem-cell division inhibitors, stipiamide, stromelysin inhibitors, sulfinosine, superactive vasoactive intestinal peptide antagonist, suradista, suramin, swainsonine, synthetic glycosaminoglycans, tallimustine, tamoxifen methiodide, tauromustine, tazarotene, tecogalan sodium, tegafur, tellurapyrylium, telomerase inhibitors, temoporfin, temozolomide, teniposide, tetrachlorodecaoxide, tetrazomine, thaliblastine, thalidomide, thiocoraline, thrombopoietin, thrombopoietin mimetic, thymalfasin, thymopoietin receptor agonist, thymotrinan, thyroid stimulating hormone, tin ethyl etiopurpurin, tirapazamine, titanocene dichloride, topotecan, topsentin, toremifene, totipotent stem cell factor, translation inhibitors, tretinoin, triacetyluridine, triciribine, trimetrexate, triptorelin, tropisetron, turosteride, tyrosine kinase inhibitors, tyrphostins, UBC inhibitors, ubenimex, urogenital sinus-derived growth inhibitory factor, urokinase receptor antagonists, vapreotide, variolin B, velaresol, veramine, verdins, verteporfin, vinorelbine, vinxaltine, vitaxin, vorozole, zanoterone, zeniplatin, zilascorb, zinostatin stimalamer.

pH-sensitive peptide-DNA complexes

In a further embodiment of the invention, the genes in plasmid DNA are brought in interaction with fusogenic peptide/NLS conjugates. In a further embodiment the NLS moiety is a stretch of histidyl residues able to assume a net

positive charge at a pH of about 5 to 6 and to show a reduction or loose completely this charge at pH above 7. The electrostatic interaction of these positively-charged peptides with the negatively-charged plasmid DNA molecules, established at pH 5-6 is weakened at physiological pH (pH-sensitive peptide-DNA complexes).

The first step of the present invention involves complex formation between the plasmid or oligonucleotide DNA with the histidyl/fusogenic peptide conjugate and lipid components in 10-90% ethanol at pH 5.0 to 6.0. The conditions must be where the histidyl residues have a net positive charge and can establish electrostatic interactions with plasmids, oligonucleotides or negatively-charged drugs. At the same time, the presence of the positively-charged lipid molecules promotes formation of micelles. At the second step, micelles are converted into liposomes by dilution with water and mixing with pre-made liposomes or lipids at pH 5-6. This is followed by dialysis against pH 7 and extrusion through membranes, entrapping and encapsulating plasmids or oligonucleotides to with a very high yield.

Whereas the composition of peptides and cationic lipids in the first step provides the lipids of the internal bilayer, the type of liposomes or lipids added at step 2 provide the external coating of the final liposome formulation (Figure 1). Examples for the formulations of peptides include: $HHHHHSPSL_{16}$ (SEQ ID NO:623), and $HHHHHSPS(LAI)_5$ (SEQ ID NO:624).

These are added at a 1:0.5:0.5 molar ratio (negative charge on DNA: cationic liposome: histidine peptide). The peptide inserts in an alpha-helical conformation inside the lipid bilayer and not only carries out DNA condensation but also endows membrane fusion properties to the complex to improve entrance across the cell membrane. The type of hydrophobic amino acids (for example, content in aromatic amino acids), in the peptide chain is very important as is the length of the peptide chain in ensuring integrity and rigidity of the complexes. Coating the outer surface of the complexes with polyethyleneglycol, hyaluronic acids and other polymers conjugated to lipids gives the particles long circulation properties in body fluids and the ability to target solid tumors and their metastases after intravenous injection, and also the ability to cross the tumor cell membrane.

Protease-sensitive linkages in peptides between the NLS and fusogenic moieties

Conversion of micelles into liposomes

An important issue of the present invention is the conversion of micelles formed between the DNA and the cationic lipids, in the presence of ethanol, into liposomes. This is done by the direct addition of the micelle complex into an aqueous solution of preformed liposomes. The liposomes have an average size of 80-160 nm or vice versa, leading to a solution of a final ethanol concentration below 10%. A formulation suitable for pharmaceutical use and for injection into humans and animals will require that the liposomes are of neutral composition (such as cholesterol, PE, PC) coated with PEG.

However, another important aspect is the research application of the present invention, such as for transfection of cells in culture. The composition of the aqueous solution of liposomes is any type of liposomes containing cationic lipids and suitable therefore for transfection of cells in culture such as DDAB:DOPE 1:1. These liposomes are pre-formed and downsized by sonication or extrusion through membranes to a diameter of 80-160 nm. The ethanolic micelle preparations are then added to the aqueous solution of liposomes with a concomitant dilution of the ethanol solution to below 10%. This step will result in further condensation of DNA or interaction of the negatively-charged phosphate groups on DNA with positively charged groups on lipids. Care must be taken so as only part of the negative charges on DNA are neutralized by lipids in the micelle. The remaining charge neutralization of the DNA is to be provided by the cationic component of the preformed liposomes in the second step.

Regulatory DNA and nuclear matrix-attached DNA

In a further embodiment of the present invention, the genes in plasmid DNA are driven by regulatory DNA sequences isolated from nuclear matrix-attached DNA using shotgun selection approaches.

The compact structural organization of chromatin and the proper spatial orientation of individual chromosomes within a cell are partially provided by the nuclear matrix. The nuclear matrix is composed of DNA, RNA and proteins and

serves as the site of DNA replication, gene transcription, DNA repair, and chromosomal attachment in the nucleus. Diverse sets of DNA sequences have been found associated with nuclear matrices and is referred to as matrix attachment regions or MARs. The MARs serve many functions, acting as activators of gene transcription, silencers of gene expression, insulators of transcriptional activity, nuclear retention signals and origins of DNA replication. Current studies indicate that different subsets of MARs are found in different tissue types and may assist in regulating the specific functions of cells. The presence of this complex assortment of structural and regulatory molecules in the matrix, as well as the in situ localization of DNA replication and transcription complexes to the matrix strongly suggest that the nuclear matrix plays a fundamental, unique role in nuclear processes. The structuring of genomes into domains has a functional significance. The inclusion of specific MAR elements within gene transfer vectors could have utility in many experimental and gene therapy applications. Many gene therapy applications require specific expression of one or more genes in targeted cell types for prolonged time periods. MARs within vectors could enhance transcription of the introduced transgene, prolong the retention of that sequence within the nucleus or insulate expression of that transgene from the expression of a cotransduced gene (reviewed by Boulikas, 1995; Bode et al, 1996).

Various biochemical procedures have been used to identify regulatory regions within genes. Traditionally, identification and selection of regulatory DNA sequences depend on tedious procedures such as transcription factor footprinting in vitro or in vivo, or subcloning of smaller fragments from larger genomic DNA sequences upstream of reporter genes. These methods have been used primarily to identify regions proximal to the 5' end of genes. However, in many instances, regulatory regions are found at considerable distances from the proximal 5' end of the gene, and confer cell type- or developmental stage- specificity. For example, studies from the groups of Grosveld and Engel (Lakshmanan et al., 1999) have shown that over 625 kb of genomic sequences surrounding the GATA-3 locus are required for the correct developmental expression of the gene in transgenic mice. Extensive DNA stretches at distances 5-20 kb upstream of the gene were found to be responsible for the central nervous system-specificity of expression. The region

between 20 to 130 kb upstream of the gene harbored regulatory regions for urogenital-specific expression of GATA-3, whereas sequences 90-180 kb downstream of the gene conferred endocardial-specific expression.

The presently disclosed method has the potential of rapidly identifying regulatory control regions. In cells, chromatin loops are formed and different attachment regions are used in different cell types or stages of development to modulate the expression of a gene. The presently disclosed method for isolating regulatory regions based on their attachment to the nuclear matrix can identify regulatory regions irrespective of their distance from the gene. Although the human genome project is expected to be almost complete by the year 2000, information on the location and nature of the vast majority of the estimated 500,000 regulatory regions will not be available.

Example 1

Plasmid DNA condenses with various agents, as well as various formulations of cationic liposomes. The condensation affects the level of expression of the reporter beta-galactosidase gene after transfection of K562 human erythroleukemia cell cultures. Liposome compositions are shown in the Table below and in FIG. 2. All lipids were from Avanti Polar Lipids (700 Industrial Park Drive, Alabaster, AL 35007). The optimal ratio of lipid to DNA was 7 nmoles total lipid/μg DNA. The transfection reagent (10 μg DNA mixed with 70 nmoles total lipid) was transferred to a small culture flask followed by the addition of 10 ml K562 cell culture (about 2 million cells total); mixing of cells with the transfection reagent was at 5-10 min after mixing DNA with liposomes. Cells were assayed for beta-galactosidase activity several times at 1-30 days post-transfection. The transfected cells were maintained in cell culture as normal cell cultures.

Best results were obtained when the cells used for transfection were at low number, not near confluence. In all experiments the transfection material was added directly in the presence of serum and antibiotics without removal of the transfection reagent or washings of the cells. This simplifies the transfection procedure and is suitable for lymphoid and other type of cell cultures that do not attach to the dish, but grow in suspension. All DNA condensing agents were purchased from Sigma. They

were suspended at 0.1 mg/ml in water. Plasmid pCMVβ was purchased from Clontech and was purified using the Anaconda kit of Althea Technologies (San Diego, CA). PolyK is polylysine, mw 9,400. PolyR is polyarginine. PolyH is polyhistidine.

To 100 µl plasmid solution (10 µg total plasmid DNA) 20 µl or 50 µl of polyK, polyR, polyH, were added; the volume was adjusted to 250 µl with water followed by addition of about 70 µl liposomes (7 nmoles /µg DNA). After incubation for 10 min to 1 h at 20°C the transfection mixture was brought in contact with the cell culture. The best DNA condensing reagent was polyhistidine compared with the popular polylysine. The best cationic lipid was DC-cholesterol (DC-CHOL: 3β [N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol). SFV is Semliki Forest virus expressing beta-galactosidase. The results are shown in FIG. 2.

Liposome	Molecular weight	Composition	Preparation
L2	DDAB mw 631 DOPE mw 744	DDAB 4.2 µmoles/ml DOPE 4.2 µmoles/ml	15 mg DDAB + 0.88 ml 20 mg/ml DOPE
L3	DOGS-NTA mw 1015.4	DOGS-NTA 1 µmole/ml DOPE 1 µmole/ml	5 mg DOGS 0.185 ml DOPE
L4	DC-Chol (mw 537) DOPE (mw 744)	DC-Chol 1 µmole/ml DOPE 1 µmole/ml	0.106 ml DC-Chol (25 mg/ml) + 0.185 ml DOPE (20 mg/ml)
L5	DOTAP (mw 698) DOPE (mw 744)	DOTAP 1.4 µmole/ml DOPE 1.3 µmole/ml	0.5 ml 10 mg/ml DOTAP + 0.25 ml DOPE (20 mg/ml)
L6	DODAP (mw 648)	DODAP 1.54 µmoles/ml DOPE 1.3 µmole/ml	0.5 ml 10 mg/ml DODAP=5 mg=7.72 µmoles + 0.25 ml DOPE (20 mg/ml)

Example 2

Targeting Genes to Tumors Using Gene Vehicles (Lipogenes).

As shown in FIG. 3, tumor targeting in SCID (severe combined immunodeficient) mice were implanted subcutaneously, at two sites, with human MCF-7 breast cancer cells. The cells were allowed to develop into large, measurable solid tumors at about 30 days post-inoculation. Mice were injected intraperitoneously with 0.2 mg plasmid pCMVβ DNA (size of the plasmid is ~4 kb)

per animal carrying the bacterial beta-galactosidase reporter gene. Plasmid DNA (200μg, 2.0 mg/ml, 0.1 ml) was incubated for 5 min with 200μl neutral liposomes of the composition 40% cholesterol, 20% dioleoylphosphatidylethanolamine(DOPE), 12% palmitoyloleoylphosphatidylcholine (POPC), 10% hydrogenated soy phosphatidylcholine (HSPC), 10% distearoylphosphatidylethanolamine (DSPE), 5% sphingomyelin (SM), and 3% derivatized vesicle-forming lipid M-PEG-DSPE.

At this stage, weak complexation of plasmid DNA with neutral (zwitterionic) liposomes takes place. This ensures homogeneous distribution of plasmid DNA to liposomes at the subsequent step of addition of cationic liposomes. After complexation of plasmid DNA with zwitterionic liposomes, 50 μl of cationic liposomes (DC-Chol 1 μmole/ml:DOPE 1.4 μmole/ml) were added and incubated at room temperature for 10 min. At this stage, a mixed liposome population is present and, most likely, formation of a type of liposome-DNA complexes containing lipids from the zwitterionic and cationic lipids takes place. The material was injected (0.35 ml total volume) to the intraperitoneal cavity of the animal. At 5 days post-injection the animal was sacrificed, the skin was removed and the carcass was incubated into X-gal staining solution for about 30 min at 37°C. The animal was incubated in fixative in X-gal staining for about 30 min (addition of 100 μl concentrated glutaraldehyde to 30 ml X-gal staining solution) and the incubation in staining solution continued. Photos were taken in a time course during the incubation period revealing the preferred organs where beta-galactosidase expression took place.

Because of the tumor vasculature targeting shown in FIG. 3E, the data imply that transfer of the genes of angiostatin, endostatin, or oncostatin to the tumors (whose gene products restrict vascular growth and inhibit blood supply to the tumor) is expected to be a rational approach for cancer treatment. Also, a combination therapy using anticancer lipogenes with encapsulated drugs into tumor targeting liposomes appears as a rational cancer therapy.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

Table 3 Simple NLS

Signal oligopeptide	Protein and features
PKKKRKV (SEQ ID NO:20)	Wild-type SV40 large T protein A point mutation converting lysine-128 (double underlined) to threonine results in the retention of large T in the cytoplasm. Transfer of this peptide to the N-terminus of β-galactosidase or pyruvate kinase at the gene level and microinjection of plasmids into Vero cells showed nuclear location of chimeric proteins.
PKKKRMV (SEQ ID NO:21)	SV40 large T with a K→M change. Site-directed mutagenesis only slightly impaired nuclear import of large T.
PKKKRKVEDP (SEQ ID NO:22)	Synthetic NLS peptide from SV40 large T antigen crosslinked to BSA or IgG mediated their nuclear localization after microinjection in *Xenopus* oocytes. The PKKGSKKA from *Xenopus* H2B was ineffective and PKTKRKV was less effective.
CGYGPKKKRKVGG (SEQ ID NO:23)	Synthetic peptide from SV40 large T antigen conjugated to various proteins and microinjected into the cytoplasm of TC-7 cells. Specified nuclear localization up to protein sizes of 465 kD (ferritin). IgM of 970 kD and with an estimated radius of 25-40 nm was retained in the cytoplasm.
CYDDEATADSQHSTPPKKK RKVEDPKDFESELLS (SEQ ID NO:24)	SV40 large T protein long NLS. The long NLS but not the short NLS, was able to localize the bulky IgM (970 kD) into the nucleus. Mutagenesis at the four possible sites of phosphorylation (double underlined) impaired nuclear import.
CGGPKKKRKVG (SEQ ID NO:25)	SV40 large T protein. This synthetic peptide crosslinked to chicken serum albumin and microinjected into HeLa cells caused nuclear localization.
PKKKIKV (SEQ ID NO:26)	A mutated (R→I) version of SV40 large T NLS. Effective NLS.
MKx11CRLKKLKCSKEKPKC AKCLKx5Rx3KTKR (SEQ ID NO:27) 74 N-terminal amino acid	Yeast GAL4 (99 kD). Fusions of the GAL4 gene portion encoding the 74 N-terminal amino acid with E. coli β-galactosidase introduced into yeast cells specify nuclear localization.
MKx11CRLKKLKCSKEKPKC A (SEQ ID NO:28) 29 N-terminal amino acid	Yeast GAL4. Acted as an efficient nuclear localization sequence when fused to invertase but not to β-galactosidase introduced by transformation into yeast cells.
PKKARED (SEQ ID NO:29) VSRKRPR (SEQ ID NO:30)	Polyoma large T protein. Identified by fusion with pyruvate kinase cDNA and microinjection of Vero African green monkey cells. Mutually independent NLS. Can exert cooperative effects.
CGYGVSRKRPRPG (SEQ ID NO:31)	Polyoma virus large T protein. This synthetic peptide crosslinked to chicken serum albumin and microinjected into HeLa cells caused nuclear localization.
APTKRKGS (SEQ ID NO:32)	SV40 VP1 capsid polypeptide (46 kD). NLS (N terminus) determined by infection of monkey kidney cells with a fusion construct containing the 5' terminal portion of SV40 VP1 gene and the complete cDNA sequence of poliovirus capsid VP1 replacing the VP1 gene of SV40.
APKRKSGVSKC (1-11) (SEQ ID NO:33)	Polyoma virus major capsid protein VP1 (11 N-terminal amino acid). Yeast expression vectors coding for 17 N-terminal amino acid of VP1 fused to β-galactosidase gave a protein that was transported to the nucleus in yeast cells. Subtractive constructs of VP1 lacking A^1 to C^{11} were cytoplasmic. This, FITC-labeled, synthetic peptide crosslinked to BSA or IgG, caused nuclear import after microinjection into 3T6 cells. Replacement of K^3 with T did not.

Signal oligopeptide	Protein and features
PNKKKRK (SEQ ID NO:34) (amino acid position 317-323)	SV40 VP2 capsid protein (39 kD). The 3' end of the SV40 VP2-VP3 genes containing this peptide when fused to poliovirus VP1 capsid protein at the gene level resulted in nuclear import of the hybrid VP1 in simian cells infected with the hybrid SV40.
EEDGPQKKKRRL (307-318) (SEQ ID NO:35)	Polyoma virus capsid protein VP2. A construct having truncated VP2 lacking the 307-318 peptide transfected into COS-7 cells showed cytoplasmic retention of VP2. The 307-318 peptide crosslinked to BSA or IgG specified nuclear import following their microinjection into NIH 3T6 cells.
GKKRSKA (SEQ ID NO:36)	Yeast histone H2B. This peptide specified nuclear import when fused to β-galactosidase.
KRPRP (SEQ ID NO:37)	Adenovirus E1a. This pentapeptide, when linked to the C-terminus of E. coli galactokinase, was sufficient to direct its nuclear accumulation after microinjection in Vero monkey cells.
CGGLSSKRPRP (SEQ ID NO:38)	Adenovirus type 2/5 E1a. This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
LVRKKRKTE$_3$SP (NLS 1) (SEQ ID NO:39) LKDKDAKKSKQE (NLS2) (SEQ ID NO:40)	*Xenopus* N1 (590 amino acid). Abundant in X. laevis oocytes, forming complexes with histones H3, H4 via two acidic domains each containing 21 and 9 (D+E), respectively. The NLS1 is required but not sufficient for nuclear accumulation of protein N1. NLS 1 and 2 are contiguous at the C-terminus.
GNKAKRQRST (SEQ ID NO:41)	v-Rel or p59 $^{v\text{-}rel}$ the transforming protein, product of the v-rel oncogene of the avian reticuloendotheliosis retrovirus strain T (Rev-T). v-Rel NLS added to the normally cytoplasmic β-galactosidase directed that protein to the nucleus.
PFLDRLRRDQK (SEQ ID NO:42) PKQKRKMAR (SEQ ID NO:43)	NS1 protein of influenza A virus, that accumulates in nuclei of virus-infected cells. Determined to be an NLS by deletion mutagenesis of NS1 in recombinant SV40. The 1st NLS is conserved among all NS1 proteins of influenza A viruses.
SVTKKRKLE (SEQ ID NO:44)	Human lamin A. Dimerization of lamin A was proposed to give a complex with two NLSs that was transported more efficiently.
SASKRRRLE (SEQ ID NO:45)	*Xenopus* lamin A. NLS inferred from its similarity to human lamin A NLS.
TKGKRKRID (SEQ ID NO:46)	*Xenopus* lamin L$_I$. NLS inferred from its sequence similarity to human lamin A NLS.
CVRTTKGKRKRIDV (SEQ ID NO:47)	*Xenopus* lamin L$_I$. This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
ACIDKRVKLD (SEQ ID NO:48)	Human c-myc oncoprotein. This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
ACIDKRVKLD (SEQ ID NO:49) (M1, fully potent NLS) RQRRNELKRSP (SEQ ID NO:50) (M2, medium potency NLS)	Human c-myc oncoprotein. Conjugation of the M1 peptide to human serum albumin and microinjection of Vero cells gives complete nuclear accumulation. M2 gave slower and only partial nuclear localization.
SALIKKKKKMAP (SEQ ID NO:51)	Murine c-abl (IV) gene product. The p160$^{gag/v\text{-}abl}$ has a cytoplasmic and plasma membrane localization, whereas the mouse type IV c-abl protein is largely nuclear.

Signal oligopeptide	Protein and features
PPKKRMRRRIE (SEQ ID NO:52) PKKKKKRP (SEQ ID NO:53)	Adenovirus 5 DBP (DNA-binding protein) found in nuclei of infected cells and involved in virus replication and early and late gene expression. Both NLS are needed, and disruption of either site impaired nuclear localization of the 529 amino acid protein.
YRKCLQAGMNLEARKTKK KIKGIQQATA (497-524 amino acid) (SEQ ID NO:54)	Rat GR, glucocorticoid receptor (795 amino acid) NLS1 determined by fusion with β-galactosidase (116 kD). NLS1 is 100% conserved between human, mouse and rat GR. Whereas the 407-615 amino acid fragment of GR specifies nuclear location, the 407-740 amino acid fragment was cytoplasmic in the absence of hormone, indicating that sequence 615-740 may inhibit the nuclear location activity. A second (NLS2) is localized in an extensive 256 amino acid C-terminal domain. NLS 2 requires hormone binding for activity.
RKDRRGGRMLKHKRQRDD GEGRGEVGSAGDMRAMIN O ACIDNLWPSPLMIKRSKK (amino acid 256-303) (SEQ ID NO:55)	Human ER (estrogen receptor, 595 amino acid) NLS. NLS is between the hormone-binding and DNA-binding regions; ER, in contrast with GR, lacks a second NLS. Can direct a fusion product with β-galactosidase to the nucleus.
RKFKKFNK (SEQ ID NO:56)	Rabbit PG (progesterone receptor). 100% homology in humans; F→L change in chickens. When this sequence was deleted, the receptor became cytoplasmic but could be shifted into the nucleus by addition of hormone; in this case the hormone mediated the dimerization of a mutant PG with a wild type PG molecule.
GKRKNKPK (SEQ ID NO:57)	Chicken Ets1 core NLS. Within a 77 amino acid C-terminal segment 90% homologous to Ets2. When deleted by deletion mutagenesis at the gene level the mutant Ets1 became cytoplasmic.
PLLKKIKQ (SEQ ID NO:58)	c-myb gene product; directs puruvate kinase to the nucleus.
PPQKKIKS (SEQ ID NO:59)	N-myc gene product; directs puruvate kinase to the nucleus.
PQPKKKP (SEQ ID NO:60)	p53; directs puruvate kinase to the nucleus.
SKRVAKRKL (SEQ ID NO:61)	c-erb-A gene product; directs puruvate kinase to the nucleus.
CGGLSSKRPRP (SEQ ID NO:62)	Adenovirus type2/5 E1a. This synthetic peptide conjugated with a bifunctional crosslinker to chicken serum albumin (CSA) and microinjected into HeLa cells directed CSA to the nucleus.
MTGSKTRKHRGSGA (SEQ ID NO:63) MTGSKHRKHPGSGA (SEQ ID NO:64)	Yeast ribosomal protein L29. Double-stranded oligonucleotides encoding the 7 amino acid peptides (underlined) and inserted at the N-terminus of the β-galactosidase gene resulted in nuclear import.
RHRKHP (SEQ ID NO:65) KRRKHP (SEQ ID NO:66) KYRKHP (SEQ ID NO:67) KHRRHP (SEQ ID NO:68) KHKKHP (SEQ ID NO:69) RHLKHP (SEQ ID NO:70) KHRKYP (SEQ ID NO:71) KHRQHP (SEQ ID NO:72)	Mutated peptides derived from yeast L29 ribosomal protein NLS, found to be efficient NLS. The last two are less effective NLS, resulting in both nuclear and cytoplasmic location of β-galactosidase fusion protein.
PETTVVRRRGRSPRRRTPSP RRRRSPRRRRSQS (SEQ ID NO:73) (One sequence, C-terminus)	Double NLS of hepatitis B virus core antigen. The two underlined arginine clusters represent distinct and independent NLS. Mutagenesis showed that the antigen fails to accumulate in the nucleus only when both NLS are simultaneously deleted or mutated.
ASKSRKRKL (SEQ ID NO:74)	Viral Jun, a transcription factor of the AP-1 complex. Accumulates in nuclei most rapidly during G2 and slowly during G1 and S. The cell cycle dependence of viral but not of cellular Jun is due to a C→S mutation in NLS of viral Jun. This NLS conjugated to rabbit IgG can mediate cell cycle-dependent translocation.

Signal oligopeptide	Protein and features
GGLCSARLHRHALLAT (SEQ ID NO:75)	Human T-cell leukemia virus Tax trans-activator protein. The most basic region within the 48 N-terminal segment. Missense mutations in this domain result in its cytoplasmic retention.
DTREKKKFLKRRLLRLDE (604-620) (SEQ ID NO:76)	Mouse nuclear Mx1 protein (72 kD), Induced by interferons (among 20 other proteins) . Selectively inhibits influenza virus mRNA synthesis in the nucleus and virus multiplication. The cytoplasmic Mx2 has R→S and R→E changes in this region.
CGYGPKKKRKV (SV40 large T) (SEQ ID NO:77) CGYGDRNKKKKE (human retinoic acid receptor) (SEQ ID NO:78) CGYGARKTKKKIK (human glucocorticoid receptor) (SEQ ID NO:79) CGYGIRKDRRGGR (human estrogen receptor) (SEQ ID NO:80) CGYGARKLKKLGN (human androgen receptor) (SEQ ID NO:81)	Synthetic peptides crosslinked to bovine serum albumin (BSA) and introduced into MCF 7 or HeLa S3 cells with viral co-internalization method using adenovirus serotype 3B induced nuclear import of BSA.
RKRQRALMLRQAR 30-42 (SEQ ID NO:82)	Human XPAC (xeroderma pigmentosum group A complementing protein) involved in DNA excision repair. By site-directed mutagenesis and immunofluorescence. NLS is encoded by exon 1 which is not essential for DNA repair function.
EYLSRKGKLEL (SEQ ID NO:83) (at the N-terminus)	T-DNA –linked VirD2 endonuclease of the Agrobacterium tumefaciens tumor-inducing (T_i) plasmid. A fusion protein with β-galactosidase is targeted to the nucleus. The T-plasmid integrates into plant nuclear DNA; VirD2 produces a site-specific nick for T integration. VirD2 also contains a bipartite NLS at its C-terminus (see Table 2).
KKSKKKRC (SEQ ID NO:84) (95-102)	Putative core NLS of yeast TRM1 (63 kD) that encodes the tRNA modification enzyme N^2, N^2-dimethylguanosine-specific tRNA methyltransferase. Localizes at the nuclear periphery. The 70-213 amino acid segment of TRM1 causes nuclear localization of β-galactosidase fusion protein in yeast cells. Site-directed mutagenesis of the 95-102 peptide resulted in its cytoplasmic retention. TRM1 is both nuclear and mitochondrial. The 1-48 amino acid segment specifies mitochondrial import.
PQSRKKLR (SEQ ID NO:85)	Max protein; specifically interacts with c-Myc protein. Fusion of 126-151 segment of Max to chicken pyruvate kinase (PK) gene, including this putative NLS, followed by transfection of COS-1 cells and indirect immunofluorescence with anti-PK showed nuclear targeting.
QPQRYGGGRGRRW (SEQ ID NO:86)	Gag protein of human foamy retrovirus; a mutant that completely lacks this box exhibits very little nuclear localization; binds DNA and RNA in vitro.

Table 4 "Bipartite" or "split" NLS

Signal Oligopeptide	Protein and features
C-terminus	*Xenopus* nucleoplasmin. Deletion analysis demonstrated the presence of a signal responsible for nuclear location.
TKKAGQAKKK (SEQ ID NO:87)	*Xenopus* nucleoplasmin
TKKAGQAKKKKLD (SEQ ID NO:88)	*Xenopus* nucleoplasmin. Whereas these 17 amino acids had NLS activity, shorter versions of the 17 amino acid sequences were unable to locate pyruvate kinase to the nucleus.
TKKAGQAKKK(KLD) (SEQ ID NO:89)	*Xenopus* nucleoplasmin. This 14 amino acid segment was identified as a minimal nuclear location sequence but was unable to locate puruvate kinase to the nucleus; three more amino acids at either end (shown in parenthesis) were needed.
CGQAKKKKLD (SEQ ID NO:90)	*Xenopus* nucleoplasmin-derived synthetic peptide; crosslinked to chicken serum albumin and microinjected to HeLa cells specified nuclear localization. This suggests that nucleoplasmin may possess a simple NLS.
KRPAMINO ACID TKKAGQAKKKK (SEQ ID NO:91)	*Xenopus* nucleoplasmin bipartite NLS. Two clusters of basic amino acids (underlined) separated by 10 amino acid are half NLS components.
HRKYEAPRHx_6PRKR (SEQ ID NO:92)	Yeast L3 ribosomal protein (387 amino acid) N-terminal 21 amino acid. Possible bipartite NLS. (Ribosomal proteins are transported to the nucleus to assemble with nascent rRNA). Fusion genes with β-galactosidase were used to transform yeast cells followed by fluorescence staining with b-gal antibody. The 373 amino acid of L3 fused to β-gal failed to localize to the nucleus, unless a 8 amino acid bridge containing a proline was inserted between L3 and β-gal.
NKKKRKLSRGSSQKTKGTSASAK ARHKRRNRSSRS (one sequence) (SEQ ID NO:93)	SV40 Vp3 structural protein. (35 amino acid C-terminus). By DEAE-dextran-mediated transfection of TC7 cells with mutated constructs.
RVTIRTVRVRRPPKGKHRK (SEQ ID NO:94)	Simian sarcoma virus v-sis gene product ($p28^{sis}$). The cellular counterpart c-sis gene encodes a precursor of the PDGF B-chain (platelet-derived growth factor). The NLS is 100% conserved between v-sis gene product and PDGF. This protein is normally transported across the ER; introduction of a charged amino acid within the hydrophobic signal peptide results in a mutant protein that is translocated into the nucleus. Puruvate kinase-NLS fusion product is transported less efficiently than cytoplasmic v-sis mutant proteins to the nucleus.
KRKIEEPEPEPKKAK (SEQ ID NO:95)	Putative bipartite NLS of *Xenopus* laevis protein factor xnf7. Inferred by similarity to the bipartite NLS of nucleoplasmin. During oocyte maturation xnf7 is cytoplasmic until mid-blastula-gastrula stage due to high phosphorylation. Partial dephosphorylation results in nuclear accumulation.
KKYENVVIKRSPRKRGRPRKD (SEQ ID NO:96)	Yeast SWI5 gene product, a transcription factor. Underlined basic amino acid show similarity to bipartite NLS of *Xenopus* nucleoplasmin. The SWI5 gene is transcribed during S, G2 and M phases, during which the SWI5 protein remains cytoplasmic due to phosphorylation by CDC28-dependent histone H1 kinase at three serine residues two near and one (double underlined) in the NLS. Translocated at the end of anaphase/G1 due to dephosphorylation of NLS. NLS confers cell cycle-regulated nuclear import of SWI5-β-galactosidase fusion protein.

Signal Oligopeptide	Protein and features
MKRKRNS 735-741 (SEQ ID NO:97) GIESIDNVMGMIGILPDMTPSTEM SMRGVRISKMGVDETSSAEKIV 449-495 (SEQ ID NO:98)	Bipartite NLS of influenza virus polymerase basic protein 2 (PB2). Mutational analysis of PB2 and transfection of BHK cells showed that both regions are involved in nuclear import. Deletion of 449-495 region gives perinuclear localization to the cytoplasmic side.
AHRARRLH (SEQ ID NO:99) 6-13 (BSI) PPRRRVRQQPP (SEQ ID NO:100) 23-33 (BSII) PARARRRRAP (SEQ ID NO:101) 39-48 (BSIII)	"Tripartite" or "doubly bipartite" NLS of adenovirus DNA polymerase (AdPol). BSI and II functioned interdependently as an NLS for the nuclear targeting of AdPol, for which BSIII was dispensable. BSII-III was more efficient NLS than BSI-II.
KRKx$_{11}$KKKSKK 207-226 (SEQ ID NO:102)	Human poly(ADP-ribose) polymerase (116 kD). The linear distance between the two basic clusters is not crucial for NLS activity in this bipartite NLS. Lysine 222 (double underlined) is an essential NLS component. DNA binding and poly(ADP-ribosyl)ating active site are independent of NLS.
GRKRAFHGDDPFGEGPPDKKGD (SEQ ID NO:103)	Herpes simplex virus ICP8 protein (infected-cell protein). This C-terminal portion of ICP8 introduced into pyruvate kinase (PK) caused nuclear targeting in transfected Vero cells. Inclusion of additional ICP8 regions to PK led to inhibition of nuclear localization.
KRPREDDDGEPSERKRARDDR (SEQ ID NO:104)	Bipartite NLS of VirD2 endonuclease of rhizogenes strains of Agrobacterium tumefaciens. Within the C-terminal 34 amino acid. Each region (underlined) independently directs β-glucuronidase to the nucleus, but both motifs are necessary for maximum efficiency. VirD2 is tightly bound to the 5' end of the single stranded DNA transfer intermediate T-strand transferred from Agrobacterium to the plant cell genome.

Table 5. "Nonpositive NLS" lacking clusters of arginines/lysines

Signal oligopeptide	Protein and features
QLVWMACNSAMIN Q ACIDFEDLRVLSFIRGTKVS PRG 327-356 (SEQ ID NO:105)	Influenza virus nucleoprotein (NP). The underlined region (327-345) when fused to chimpanzee a_1-globin at the cDNA level and microinjected into *Xenopus* oocytes specifies nuclear localization.
MNKIPIKDLLNPQ (NLS1 at N-terminus) (SEQ ID NO:106) VRILESWFAKNIEN PYLDT (NLS2 at amino acid 141-159, part of the homeodomain) (SEQ ID NO:107)	Yeast MAT a2 repressor protein, containing a homeodomain. The two NLS are distinct, each capable of targeting β-galactosidase to the nucleus. However, deletion of NLS2 results in a2 accumulation at the pores. NLS1 and 2 may act at different steps in a localization pathway. Part of the homeodomain mediates nuclear localization in addition to DNA binding. The core pentapeptide containing proline and two other hydrophobic amino acids flanked by lysines or arginines (underlined) was suggested as one type of NLS core.
$Rx_7Kx_{15}KIPRx_3HFY$ EERLSWYSDNED (SEQ ID NO:108) 152-206 (C-terminal segment)	*Drosophila* HP1 (206 amino acids) that binds to heterochromatin and is involved in gene silencing. NLS identified by β-galactosidase/HP1 fusion proteins introduced by P-element mediated transformation into *Drosophila* embryos.
FVx_{7-} $_{20}MxSLxYMx_4MF$	Adenovirus type 5 E1A internal, developmentally-regulated NLS. This NLS functions in *Xenopus* oocytes but not in somatic cells. This NLS can be utilized up to the early neurula stage.

Table 6. Nucleolar localization signals (NoLS)

Signal oligopeptide	Protein and features
MPKTRRRPRRSQRKRPPTP (SEQ ID NO:109)	Nucleolus localization signal in amino terminus of human $p27^{x-III}$ protein (also called Rex) of T cell leukemia virus type I (HTLV-I). When this peptide is fused to N-terminus of β-galactosidase, directs it to the nucleolus. Deletion of residues 2-8 (underlined), 12-18 (double-underline) or substitution of the central RR (dotted-underlined) with TT abolish nucleolar localization. Other amino acids between positions 20-80 increase nucleolar localization efficiency.
RLPVRRRRRRVP (SEQ ID NO:110)	Adenovirus pTP1 and pTP2 (preterminal proteins, 80 kD) between amino acid residues 362-373. The 140 kD DNA polymerase of adenovirus when it has lost its own NLS can enter the nucleus via its interaction with pTP. The staining was nuclear and nucleolar with some perinuclear staining as well. The NLS fused to the N-terminus of E. coli β-galactosidase was functional in nuclear targeting.
GRKKRRQRRRP (SEQ ID NO:111)	HIV (human immunodeficiency virus) Tat protein; localizes pyruvate kinase to the nucleolus. Tat is constitutively nucleolar.
RKKRRQRRR(AHQ) Nucleolar localization signal (SEQ ID NO:112)	Tat positive trans-activator protein of HIV-1 (human immunodeficiency virus type 1). The 3 amino acids shown in parenthesis are essential for the localization of the β-galactosidase to the nucleolus. The 9 amino acid basic region is able to localize β-gal to the nucleus but not to the nucleolus.
KRVKLDQRRRP (SEQ ID NO:113)	Artificial sequence from c-Myc and HIV Tat NLSs that effectively localizes pyruvate kinase to the nucleolus.
FKRKHKKDISQNKRAVRR (SEQ ID NO:114)	Human HSP70 (heat shock protein of 70 kD); localizes pyruvate kinase to the nucleus and nucleolus. HSP70 is physiologically cytoplasmic but with heat-shock HSP70 redistributes to the nucleoli, suggesting that the nucleolar targeting sequence is cryptic at physiological temperature and is revealed under heat-shock.
RQARRNRRRRWRERQR (35-50) (SEQ ID NO:115)	HIV-1 Rev protein (116 amino acid, nucleolar). Mutations in either of the two regions of arginine clusters severely impair nuclear localization. β-galactosidase fused to R_4W was targeted to the nucleus, and fused to the entire 35-50 region, was targeted to the nucleolus.
RQARRNRRRRWRERQRQ (35-51) (SEQ ID NO:116)	HIV-1 Rev protein. A fusion of this Rev peptide with β-galactosidase became nuclear but not nucleolar. The 1-59 amino acid segment of Rev fused to β-galactosidase localized entirely within the nucleolus. Whereas the NRRRRW (bold) is responsible for nuclear targeting, the RR and WRERQRQ (double underlined) specify nucleolar localization. Rev may function to export HIV structural mRNAs from the nucleus to the cytoplasm.

Table 7. Karyophilic clusters on non-membrane protein kinases

Karyophilic peptides	Non-membrane protein kinase	Species	Features
73 FVVHKRCHE (SEQ ID NO:117) 96 DDPRSKHKFKIH (SEQ ID NO:118) 577 TKHPGKRLG (SEQ ID NO:119)	Protein kinase C (673 aa)	Bovine, human β type	Known to translocate to the nucleus following treatment of cells with mitogens.
71 FVVHRRCHEF (SEQ ID NO:120) 95 DDPRNKHKFRLH (SEQ ID NO:121) 591 TKHPAKRLG (SEQ ID NO:122)	Protein kinase C (697 aa)	bovine, human γ type	
72 FVVHKRCHE (SEQ ID NO:123) 96 DDPRSKHKFKIH (SEQ ID NO:124) 577 TKHPGKRLG (SEQ ID NO:125)	Protein kinase C (673 aa)	rabbit type α and β	
71 FVVHRRCHE (SEQ ID NO:126) 95 DDPRNKHKFRLH (SEQ ID NO:127) 594 TKHPGKRLG (SEQ ID NO:128)	PKC-I (701 aa)	rat brain	
22 GENKMKSRLRKG (not conserved) (SEQ ID NO:129) 80SYVVHKRCHEYVT (conserved) (SEQ ID NO:130) 211PDDKDQSKKKTR TIK (not conserved) (SEQ ID NO:131) 614PPFKPKIKHRKMC P (not conserved) (SEQ ID NO:132)	Protein kinase C (639aa, 75 kDa)	*Drosophila*	14 exons, 20 kb; 3 transcripts in adult flies; not expressed in 0-3h *Drosophila* embryos; the **VVHKRCHE** (SEQ ID NO:133)motif (or **VVHRRCHE** (SEQ ID NO:134)) is conserved among all PKC known.
148 KKVLQDKRFK NRELQIMRKLD (SEQ ID NO:135)	Glycogen synthase kinase 3 GSK-3α (483 aa) GSK-3β (420 aa)	rat brain	Phosphorylates glycogen synthase, c-Jun, c-Myb; two isoforms encoded by discrete genes; highly expressed in brain; both α and β forms are cytosolic but also associated with the plasma membrane consistent with their role in signal transduction from the cell surface.
LQDRRFKNRELQ (SEQ ID NO:136)	Zw3 zeste-white 3	*Drosophila*	Product of the segment polarity gene zw3; the protein encoded has 34% homology to cdc2; mutations in zw3 give embryos that lack most of the ventral denticles, differentiated structures derived from the most anterior region of each segment.

Karyophilic peptides	Non-membrane protein kinase	Species	Features
289ECLKKFNARRKL KGAIL (SEQ ID NO:137)	Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) β subunit (542aa, 60.3 kDa)	rat brain	Composed of nine 50 kDa α-subunits and three 60 kDa β-subunits; both are catalytic; calmodulin- and ATP-binding domains; highly expressed in forebrain neurons, concentrated in postsynaptic densities; acts as a Ca^{2+}-triggered switch and could be involved in long-lasting changes in synapses.
290LKKFNARRKL KGAILTTM (SEQ ID NO:138) 450EETRVWHRRDGK (SEQ ID NO:139)	CaM kinase II (478 aa, 54 kDa) α-subunit	rat brain	This particular isoform is exclusively expressed in the brain; high enzyme levels in specific brain areas; might be involved in short- and long-term responses to transient stimuli.
185 GFAKRVKGRT WTLCG (SEQ ID NO:140)	CADPK catalytic subunit (349 aa, 40.6 kDa)	bovine (cardiac muscle)	By Edman degradation of protein fragments; mediates the action of and is activated by cAMP; consists of two regulatory (R) and two catalytic (C) subunits; cAMP releases the C subunit from the inactive R_2C_2 cADPK; two cDNAs were cloned encoding two isoforms of the catalytic subunit of cADPK in mouse.
186 GFAKRVKGRTW TLCG (SEQ ID NO:141)	CADPK (catalytic subunit) (350 aa)	bovine	cDNA was isolated by screening a bovine pituitary cDNA library; 93% sequence similarity to known bovine cADPK; represents the second gene for the catalytic subunit of cADPK.
29 EEEIQELKRKLH KCQSVLP (SEQ ID NO:142) 389 KILKKRHIVDTR (SEQ ID NO:143)	CGDPK (SEQ ID NO:144) (670 aa, 76.3 kDa)	bovine lung	By protein sequencing; composed of two identical subunits activated in an allosteric manner by binding of cGMP and not by dissociation of catalytic subunit as in cADPK; sequence similar to cADPK
117 KTLKKHTIVK (SEQ ID NO:145)	TPK3 (398 aa) cADPK	*S. cerevisiae*	cAMP-DPK is a tetrameric protein with two catalytic and two regulatory subunits; cAMP activates the kinase by dissociating the catalytic subunits from the tetramer; all three TPK 1, 2, 3 are catalytic subunits.
$16S_2H_{13}GHG_2$ 166 EYCHRHKIVHRD LKP (SEQ ID NO:146) 495 PLVTKKSKTRWH FG (SEQ ID NO:147)	SNF1 (633aa, 72 kDa)	*S. cerevisiae*	Ser/Thr kinase; autophosphorylated; plays a central role is carbon catabolite repression in yeast required for expression of glucose-repressible genes; region 60-250 shows high sequence similarity to cAMP-dependent protein kinase (cADPK).

Karyophilic peptides	Non-membrane protein kinase	Species	Features
70 PVKKKKIKREIK (SEQ ID NO:148) 269 DILQRHSRKRW ERF (SEQ ID NO:149) 146 PKSSRHHHTDG (SEQ ID NO:150)	Casein kinase II (α-subunit, catalytic) (336aa) CKII (β-subunit, regulatory) (215aa)	*Drosophila melanogaster* *Drosophila melanogaster*	CKII is composed of α and β subunits in a $\alpha_2\beta_2$ 130-150 kDa protein; the α-subunit is the catalytic and the β is autophosphorylated.
142 PKSSRHHHTDG (SEQ ID NO:151)	CKII (β-subunit, regulatory) (209aa, 24.2 kDa)	bovine (lung)	
108 PKQRHRKSLG (SEQ ID NO:152) 129 GSMCKVKLAK HRYTNE (SEQ ID NO:153) 506 DRKHAKIRNQ (SEQ ID NO:154) 638 GNIFRKLSQRR KKTIEQ (SEQ ID NO:155) 773 PPLNVAKGRKL HP (SEQ ID NO:156)	KIN1 (1064 aa, 117 kDa)	*S. cerevisiae*	30% aa similarity to bovine cADPK and 27% (KIN1) or 25% (KIN2) aa similarity to v-Src within the kinase domain; the catalytic domains of KIN1 and KIN2 are near the N-terminus and are structural mosaics with features characteristic of both Tyr and Ser/Thr kinases.
87 ELRQFHRRSLG (SEQ ID NO:157) 111 GKVKLVKHRQ TKE (SEQ ID NO:158) 217 GSLKEHHARKF ARG (SEQ ID NO:159) 807 LSVPKGRKLHP (SEQ ID NO:160)	KIN2 (1152 aa, 126 kDa)	*S. cerevisiae*	
60FLRRGIKKKLTLD (SEQ ID NO:161) 472 PSKDDKFRHWC RKIKSKIKEDKRIKRE (SEQ ID NO:162)	STE7 (515 aa)	*S. cerevisiae*	Implicated in the control of the three cell types in yeast: (a, α , and a/α) of which a and α cells are haploid and are specialized for mating whereas a/α cells are diploid and are specialized for meiosis and sporulation; with the exception of the mating type locus, MAT, all cells contain the same DNA sequences. STE7 gene produces insensitivity to cell-division arrest induced by the yeast mating hormone, α-factor.
722 QRRVKKLPSTTL (SEQ ID NO:163) QRRVKKLPSITL (SEQ ID NO:164)	S6KIIα (733aa) S6KII β	*Xenopus* *Xenopus*	
742 QRRVKKLPSTTL (SEQ ID NO:165)	S6KII (752 aa)	Chicken	
713QRRVRKLPSTTL (SEQ ID NO:166)	S6KII (724aa)	Mouse	

Karyophilic peptides	Non-membrane protein kinase	Species	Features
16GVVYKGRHKTTG (SEQ ID NO:167) 120 FCHSRRVLHRD LKP (SEQ ID NO:168)	CDC2Hs (297aa) $p34^{cdc2}$	Human	Isolated by expressing a human cDNA library in *S. pombe* and selecting for clones that complement a mutation in the cdc2 yeast gene; the human CDC2 gene can complement both the inviability of a null allele of *S. cerevisiae* CDC28 and cdc2 mutants of *S. pombe*; CDC2 mRNA appears after that of CDK2.
GVVYKARHKLSGR (SEQ ID NO:169)	cdc2 (297aa)	*S. pombe*	High homology to *S. cerevisiae* CDC28.
119HSHRVLHRDLKP (SEQ ID NO:170)	CDK2 (cell division kinase 2) (298 aa)	Human	The human CDK2 protein has 65% sequence identity to human $p34^{cdc2}$ and 89% sequence identity to *Xenopus* Eg1 kinase; human CDK2 was able to complement the inviability of a null allele of *S. cerevisiae* CDC28 but not cdc2 mutants in *S. pombe*. CDK2 mRNA appears in late G1/early S.
109 FCHSHRVLHRD LKP (SEQ ID NO:171)	Eg1 (297aa)	*Xenopus*	Cdk2-related
125 GIAYCHSHRILH RDLKP (SEQ ID NO:172)	CDC28 (298a)	*S. cerevisiae*	The homolog of *S. pombe* Cdc2
119 HSHRVIHRDLKP (SEQ ID NO:173)	cdk3 (305aa)	Human	
56 KELKHKNIVR (SEQ ID NO:174)	PSSALRE (291 aa) (SEQ ID NO:175)	Human	cdc2 -related kinase.
1 MDRMKKIKRQ (N-terminus) (SEQ ID NO:176) 141 DKPLSRRLRRV (SEQ ID NO:177)	PCTAIRE-1 (496 aa)	Human	cdc2-related kinase.
1 MKKFKRR (SEQ ID NO:178) 129 RNRIHRRIS (SEQ ID NO:179) 172 SRRSRRAS (SEQ ID NO:180) 304 HRRKVLHR (SEQ ID NO:181) 512 GHGKNRRQSM LF (SEQ ID NO:182)	PCTAIRE-2 (523 aa)	Human	cdc2 related kinase.
163 HTRKILHR (SEQ ID NO:183) 369 PGRGKNRRQSIF (SEQ ID NO:184)	PCTAIRE-3 (380 aa)	Human	cdc2 related kinase.

Karyophilic peptides	Non-membrane protein kinase	Species	Features
69 EVFRRKRRLH (SEQ ID NO:185) 302 DKPTRKTLRKSR KHH (SEQ ID NO:186)	KKIALRE (358 aa) (SEQ ID NO:187)	Human	cdc2-related kinase.
1 MVKRHKNT (SEQ ID NO:188) 87 DGELFHYIRKHGP (SEQ ID NO:189) 114 DAVAHCHRFRFR HRD (SEQ ID NO:190) 295 KKSSSKKVVRRL QQRDD (SEQ ID NO:191)	nim1$^+$ gene product (new inducer of mitosis); protein kinase (370 aa)	*S. pombe*	
194 PAQKLRKKNNFD (SEQ ID NO:192) 388 KQHRPRKNTNFT PLPP (SEQ ID NO:193) 592 KYAVKKLKVKF SGP (SEQ ID NO:194)	Wee1$^+$ gene product (877aa)	*S. pombe*	The Wee1$^+$ gene functions as a dose-dependent inhibitor that delays the initiation of mitosis until the yeast cell has attained a certain size; Wee1 has a protein kinase consensus probably regulating cdc2 kinase.
266 PNETRRIKRAN RAG (SEQ ID NO:195)	CDC7 (497 aa)	*S. cerevisiae*	Required for mitotic but not meiotic DNA replication presumably to phosphorylate specific replication protein factors; implicated in DNA repair and meiotic recombination; some homology with CDC28 and oncogene protein kinases but differs in a large region within the phosphorylation receptor domain.
48YDHVRKTRVAIKK (SEQ ID NO:196)	ERK1 (MAP kinase) (367 aa; 42 kDa)	Rat	Known to translocate to the nucleus following their activation by phosphorylation at T-190, and Y-192 (T-183, Y-185 in ERK2).
59 ILKHFKHE (SEQ ID NO:197)	FUS3 (353aa)	*S. cerevisiae*	MAP-(ERK1)-related.
252 QIKSKRAKEY (SEQ ID NO:198)	KSS1 (368 aa)	*S. cerevisiae*	MAP-(ERK1)-related.
ELVKHLVKHGSN (SEQ ID NO:199) GKAKKIRSQLL (SEQ ID NO:200)	SWI6 (803aa, 90kDa)	*S. cerevisiae*	Activator of CACGA-box with sequence similarity to cdc10; required at START of cell cycle.
EQRLKRHRIDVSDED (SEQ ID NO:201) SNIKSKCRRVV (SEQ ID NO:202)	cdc10	*S. pombe*	
37 PPKRIRTD (suggested by the authors) (SEQ ID NO:203) 492 KLARKQKRP (SEQ ID NO:204)	CTD kinase (528 aa) 58 kDa subunit (catalytic)	*S. cerevisiae*	Consists of 3 subunits of 58, 38, and 32 kDa; disruption of the 58 kDa gene gives cells that lack CTD kinase, grow slowly, are cold sensitive, but have different phosphorylated forms of RNA pol II.

Karyophilic peptides	Non-membrane protein kinase	Species	Features
29 GVSSVVRRCIHKP (SEQ ID NO:205)	Phosphorylase kinase (catalytic subunit) (386aa)	Rabbit (skeletal muscle)	
489 KKYMARRKW QKTGHAV (SEQ ID NO:206)	Myosin light chain kinase (MLCK) (669 aa)	Chicken gizzard	Ca^{2+}/calmodulin-activated; phosphorylated by cADPK; first described as responsible for the phosphorylation of a specific class of myosin light chains; required for initiation of contraction in smooth muscle.
314 PWLNNLAEKAK RCNRRLKSQ (SEQ ID NO:207) 334 ILLKKYLMKRR WKKNFIAVS (SEQ ID NO:208)	Myosin light chain kinase (partial 368 carboxy-terminal aa sequence)	Rabbit (skeletal muscle)	By protein sequencing.
28 GVSSVVRRCIHKP (SEQ ID NO:209)	Phosphorylase kinase (PhK) (catalytic γ subunit) (389 aa)	Mouse (muscle)	Glycogenolytic regulatory enzyme; undergoes complex regulation; composed of 16 subunits containing equimolar ratios of α, β, γ and δ subunits; high levels in skeletal muscle; isoforms in cardiac muscle and liver; cDNA probe does not hybridize to X chromosome in mice and is thus distinct from the mutant recessive PhK deficiency that results in glycogen storage disease.

Table 8. Nuclear localization signals on DNA repair proteins

Putative NLS	Gene product	Equivalent protein in other species	Features
HIGHER EUKARYOTES			
None (N-terminus) MDPGKDKEGvpqpsgppaRKKF (bipartite NLS) (SEQ ID NO:210)	ERCC1	RAD10	297aa; DBD; interacts strongly with ERCC4 (XPF) to form an excision endonuclease; unless the KDKx$_{11}$RKK is a bipartite NLS it may depend upon its binding with ERCC4 for its nuclear import.
None 681DKRFARGDKRGKLPR (near the C-terminus) (four positive , one negative over a heptapeptide stretch) (SEQ ID NO:211)	ERCC2 (XPD)	RAD3 (S. cer)	760 aa; DNA helicase component of TFIIH, essential for cell viability; contains one nucleotide-binding, one DNA-binding, and seven domains characteristic of helicases; 52% identity with S. cer RAD3 at the amino acid level.
8 DRDKKKSRKRHYEDEE (SEQ ID NO:212) 522 YVAIKTKKRILLYTM (SEQ ID NO:213) (weak NLS if at all, hydrophobic environment) 769 PSKHVHPLFKRFRK (SEQ ID NO:214)	ERCC3 (XPB)	SSL2 (S cer) Haywire(Dros)	782 aa; helicase, component of TFIIH essential for cell viability; helix-turn-helix, DNA-BD, and helicase domains
84 KKQTLVKRRQRKD (SEQ ID NO:215) 210 EFTKRRRTL (SEQ ID NO:216) 390 DESMIKDRKDRLP (SEQ ID NO:217) 1170 GKKRRKLRRARGRK RKT (SEQ ID NO:218)	ERCC5 (XPG)	RAD2; Rad13	1186 aa in human, 1196 in X. laevis; 3' incision endonuclease; involved in homologous recombination; strongly nuclear
253PQKQEKKPRKIMLNEASG (SEQ ID NO:219) 314 PNKKARVLSKKEERLKK HIKKLQKR (SEQ ID NO:220) 406 PLPKGGKRQKKVP (SEQ ID NO:221) 455 DGDEDYYKQRLRRWNK LRLQDKEKRLKLEDDSEESD (SEQ ID NO:222) 1028 DVQTPKCHLKRRIQP X$_8$PKRKKFP (SEQ ID NO:223) 1180 KHKSKTKHHSVAEEETL EKHLRPKQKPKX$_{15}$PHLVKK RRY (SEQ ID NO:224) 1324 PAGKKSRFGKKRN (SEQ ID NO:225)	ERCC6 CS-B	RAD26	1493aa; involved in the preferential repair of active genes; nonessential for cell viability

Putative NLS	Gene product	Equivalent protein in other species	Features
21 PASVRASIERKRQRALM LRGAR (SEQ ID NO:226) 160 PPLKFIVKKNPHHSQW GD (weak) (SEQ ID NO:227) 210 NREKMKQKKFDKKVKE (weak because of F) (SEQ ID NO:228)	XPA	RAD14	273 aa; zinc finger domain; involved in lesion recognition
72 YLRRAMKRFN (weak) (SEQ ID NO:229) 262 PSAKGKRNKGGRKKRSK PSSSEEDEGPG (SEQ ID NO:230) 297 QRRPHGRERR (weak) (SEQ ID NO:231) 368 RTHRGSHRKDP (weak) (SEQ ID NO:232) 384 SSSSSSSKRGKKMCSDG (SEQ ID NO:233) 531 ALKRHLLKYE (weak) (SEQ ID NO:234) 594 SNRARKARLAEP (SEQ ID NO:235) 660 PNLHRVARKLD (weak) (SEQ ID NO:236) 716 ERKEKEKKEKR (SEQ ID NO:237) 740 IRERLKRRYG (SEQ ID NO:238) 801 GGPKKTKRERK (SEQ ID NO:239)	XPC	RAD4 (23% identity, 44% similarity)	823 aas, 92.9 kDa; very hydrophilic protein; might be involved in lesion recognition since XPC cells (40% of all XP cases) can repair active parts of the genome whereas inactive and the nontranscribed strand of active genes are not repaired
20 KSKAKSKARREEEEED (SEQ ID NO:240) 54 GKRKRG (SEQ ID NO:241) 69 GPAKKKVAKVTVK (SEQ ID NO:242) 103 PSDLKKAHHLKRG (SEQ ID NO:243)	XPC		940 aa; the first 117 aa are lacking in the Legerski and Peterson, (1992) XPC sequence (see above); the following 823aa are identical.
82 EIDRRKKRPLENDGPVKK KVKKVQQKE (SEQ ID NO:244) 375 KENVRDKKKG (SEQ ID NO:245) 571 FGRRKLKKWVT (SEQ ID NO:246) 710 PLIKKRKDEIQG (SEQ ID NO:247) 1091 KELEGLINTKRKRLKYF AKLW (SEQ ID NO:248)	Rep-3 (mouse) Duc-1 (HeLa)	Swi4 (*S pom*)	1137aa; mismatch repair protein; Rep-3 is in the immediate 5' flanking region of DHFR gene (89 bp) but transcribed from the opposite strand; a bidirectional promoter is used for both transcripts.
422 EKHEGKHQKLL (weak) (SEQ ID NO:249)	hMSH2	MSH2 (*S cer*)	human mismatch repair protein; homologous to *S. cerevisiae* MSH2; associated with the hereditary nonpolyposis colon cancer gene on chromosome 2p16.

Putative NLS	Gene product	Equivalent protein in other species	Features
397 PDIRRLTKKLNKRG (SEQ ID NO:250) 547 DAKELRKHKKYIE (SEQ ID NO:251) 869 VKMAKRKANE (SEQ ID NO:252)	MSH2 (S cer)		
95 GELAKRSERRAEAE (SEQ ID NO:253) 354 KRKEPEPKGSTKKKAK TG (SEQ ID NO:254) 394 GKFKRGK (SEQ ID NO:255)	Human Rad2	Rad2 (*S. pom*)	400 aa; required for fidelity of chromosome separation at mitosis; limited similarity to RAD2 (ssDNA nuclease), rad13, and XPG (ERCC5).
None	mouse RAD51		339 aa; recombination-repair protein; 83% homology to S cerevisiae RAD51 and 55% homology to *E. coli* RecA.
None	HHR23B /p58	RAD23	Subunit of XPC (125 kDa)
None	HHR23A	RAD23	Subunit of XPC (125 kDa)
32 PSQAEKKSRARAQ (SEQ ID NO:256)	RPA (34 kDa subunit)		RPA (70, 34, and 14 kDa subunits) might stabilize the helicase-melted DNA around the lesion; antibodies against RPA 32 kDa subunit inhibit DNA replication.
GAKKRKIDDA (SEQ ID NO:257)	ATPase Q1	RecQ (*E. coli*)	649 aa; altered in XPC cells; undetermined role in repair
PKKPRGKM (SEQ ID NO:258) EHKKKHP (SEQ ID NO:259) ETKKKFKDP (SEQ ID NO:260) EKSKKKK(E/D)$_{41}$ (SEQ ID NO:261) E_3G_2KKKKKFAK (SEQ ID NO:262)	HMG-1		Calf thymus HMG 1 (259 aa); involved in the recognition of cisplatin lesions
512 RDEKKRKQLKKAKAK MAKDRKSRKKP (SEQ ID NO:263) 619 GESSKRDKSKKKKKVKV KMEKK (SEQ ID NO:264) 674 GENKSKKKRRRSEDSEE EE (SEQ ID NO:265)	SSRP1	ABF (*S cer*)	709 aa, 81 kDa, structure-specific recognition protein 1; involved in recognition of cisplatin-induced lesions; also involved in Ig gene recombination; one HMG-box, similarity to SRY, MTFII, LEF-1, TCF-1a, and ABF2.
1 MPKRGKKG (SEQ ID NO:266)	Ref-1 (HAP1)		Redox factor 1 from HeLa cells; 37 kDa, 318 aa; apurinic/apyrimidinic (AP) endonuclease for DNA repair but also of redox activity stimulating Jun/Fos DNA binding.
1 MPKRGKKG (SEQ ID NO:267)	HAP1 (bovine)	ExoIII (*E. coli*) ExoA (*S. pneumoniae*)	323 aa; apurinic/apyrimidinic (AP)-endonuclease

Putative NLS	Gene product	Equivalent protein in other species	Features
DROSOPHILA			
1 MGPPKKSRKDRSGGDKF GKKRRGQDE (SEQ ID NO:268) EMSYSRKRQRFLVNQG (weak) (SEQ ID NO:269) YYEHRKKNIGSVHPLFK KFRG (bipartite) (SEQ ID NO:270)	Haywire	ERCC3 (XPB) SSL2 (S cer)	helicase with 66% identity to human ERCC3; flies expressing marginal levels of Haywire display motor defects and reduced life span
77 ARGKKKQPK (SEQ ID NO:271) 98 KPKGRAKKA (SEQ ID NO:272) 157 QAKGRKKKELP (SEQ ID NO:273) 179 EPPKQRARKE (SEQ ID NO:274) 241 PPKAASKRAKKGK (SEQ ID NO:275) 282 PKKRAKKTT (SEQ ID NO:276) 317 EPAPGKKQKKSAD (SEQ ID NO:277) 336 EEEAKPSTETKPAKGR KKAP (SEQ ID NO:278) 372 KPARGRKKA (SEQ ID NO:279) 394 GSKTTKKAKKAE (SEQ ID NO:280)	Rrp1	HAP1	Recombination repair protein 1); 679 aa; the 252 aa C-terminal domain is homologous to AP-endonucleases, whereas the 1-426 aa domain is highly charged, carries all of the putative NLSs.
S. CEREVISIAE			
200 IEKRRKLYISGG (SEQ ID NO:281) 515 NKKRGVRQVLLN (SEQ ID NO:282) 565 KEQVTTKRRRTRG (conserved in Rad16) (SEQ ID NO:283) 1024 NLRKKIKSFNKLQ (SEQ ID NO:284)	RAD1	ERCC4 (XPF) Rad16	1100 aa; 30% sequence identity to Rad16; RAD1 interacts strongly with RAD10
89 RQRKERRQGKRE (SEQ ID NO:285) 907 ENKFEKDLRKKLVNNE (SEQ ID NO:286) 984 RDVNKRKKKGKQKRI (SEQ ID NO:287) 1017 KRISTATGKLKKRKM (SEQ ID NO:288)	RAD2	XPGC Rad13	1031 aa, 117.8 kDa; ssDNA endonuclease; rad mutants are defective in incision
672 GKDDYGVMVLADRRF SRKRSQLP (contains the bulky F) (SEQ ID NO:289)	RAD3 (S. cer)	ERCC2 or XPD; Rad15 or Rhp3	778 aa, 89,779 Da; 30% sequence identity to rad16; ATP-dependent DNA helicase; single-stranded DNA-dependent ATPase.

Putative NLS	Gene product	Equivalent protein in other species	Features
26 PLSRRRRVRRKNQPLPD AKKKFKTG (SEQ ID NO:290) 134 NEERKRRKYFHMLYL (SEQ ID NO:291) 160 EWINSKRLSRKLSNL (weak) (SEQ ID NO:292) 254 EMSANNKRKFKTLKRSD weak (SEQ ID NO:293) 382 WMNSKVRKRRITKDDF GEK (SEQ ID NO:294) 403 RKVITALHHRKRTKID DYED (SEQ ID NO:295) 504 KTGSRCKKVIKRTVGRP (SEQ ID NO:296)	RAD4	XPC	754 aa; mutations in RAD4 that that inactivate the excision repair function of RAD4 result in truncated proteins missing the C-terminal one-third of RAD4.
150 FHPKRRRIYGFR (SEQ ID NO:297) 215 DSRGRKKASM (SEQ ID NO:298) 297 DGESLMKRRRTEGGNK REK (SEQ ID NO:299) 1152 DEDERRKRRIEE (SEQ ID NO:300)	RAD5		1169 aa; helicase involved in postreplication-repair (RAD6 epistasis group); binds DNA with the seven helicase motifs and with zinc fingers; increases the instability of poly (GT) repeats in the yeast genome.
1 MSTPARRRLMRDFKRM KEDAPP (SEQ ID NO:301)	RAD6		RAD6 mediates the ubiquitination of H2A and H2B histones
15 GVAKLRKEKSGAD (SEQ ID NO:302) 76 DDYNRKRPFRSTRPGK (SEQ ID NO:303)	RAD10	ERCC1	210 aa; forms an endonuclease with RAD1; the basic and tyrosine-rich central domain was suggested to bind DNA by ionic interactions and tyrosine intercalation.
172 EGKAHRREKKYE (SEQ ID NO:304) 200 NRLREKKHGKAHIHH (SEQ ID NO:305)	RAD14	XPAC	247aa, 29.3 kDa; two zinc fingers; involved in lesion recognition; 27% sequence identity and 54% sequence similarity (if conserved residues are grouped together) to human XPA; deletion of RAD14 gene generates high UV sensitivity.
345 ERRKQLKKQGPKRP (SEQ ID NO:306) 479 ETYKKRIKEWESCYPDE (SEQ ID NO:307)	Ixr1 (*S. cer*)		591 aa; two consecutive HMG boxes; involved in recognition of 1,2-intrastrand d(GpG) and d(ApG) cisplatin crosslinks.
None	RAD23	HHR23	

Putative NLS	Gene product	Equivalent protein in other species	Features
483 LTCKKLKTHNRIILSG weak (SEQ ID NO:308) 934 NALRKSRKKITKQYEIGT PX_9GEIRKRDP (SEQ ID NO:309)	RAD26 (yeast ERCC6)	ERCC6 CS-B (hum)	1075aa; disruption of the RAD26 gene gives viable yeast cells unable to preferentially repair the actively transcribed strands; surprisingly, in contrast to human CS-B cells, disruption of the RAD26 in yeast does not cause sensitivity to UV, Cisplatin, or X-rays.
634 KPTSKPKRVRTATKKKIP (SEQ ID NO:310) 408 FYKKRSPVTRSKKSG (SEQ ID NO:311)	MRE11	Rad32 (S pom)	meiotic recombination protein; functions in the same pathway with RAD51
none; 361 GFKKGKGCQR (SEQ ID NO:312)	RAD51	RecA (*E. coli*)	402 aa; essential for repair of DSBs and recombination; associates strongly with RAD52; self associates; neither RAD51 nor RAD52 possess a typical simple NLS.
none; 328 GFKKGKGCQR (SEQ ID NO:313)	RAD51 (K. lactis)		364 aa
none; 155 ERAKKSAVTDALKRSLR GFGX8DKDFLAKIDKVKFDP PD (tripartite) (SEQ ID NO:314)	RAD52	Rad22	504 aa; rad52 mutants are defective in ionizing radiation, mitotic recombination, mating-type switching, and repair of DSDs.
1 MARRRLPDRPP (SEQ ID NO:315) 65 GGRSLRKRSA (SEQ ID NO:316) 99 QLTKRRKD (SEQ ID NO:317)	RAD54		898 aa; recombination-repair protein; ATP-binding motif; helicase domains; in the same subfamily of helicases with MOT1 and SNF2.
269 DETVFVKSKRVKASSS (extremely weak if at all NLS) (SEQ ID NO:318) 317 GEDRKREGRNLKR (SEQ ID NO:319)	RAD55		Similarity to RecA, and lower similarity to RAD51, RAD57, and DMC1
371 PISRQSKKRKFDYRVP (SEQ ID NO:320)	RAD57		460 aa; nucleotide-binding domain; limited similarity to RAD51
62 GLKKPRKKTKSSRH (SEQ ID NO:321) 688 GRILRAKRRNDEG (SEQ ID NO:322) 784 GRGSNGHKRFKS (weak) (SEQ ID NO:323)	SSL2	ERCC3 (XPB)	843 aa; putative helicase that seems to function in repair but also in the removal of secondary structures in the 5' untranslated region of mRNA to allow ribosome binding and scanning.

Putative NLS	Gene product	Equivalent protein in other species	Features
50 TRRHLCKIKGLSE (weak) (SEQ ID NO:324) 277 DGRKPIGGHX$_{12}$RKGRG DER (bipartite) (SEQ ID NO:325)	DMC1	RecA	334 aa; yeast homolog of RecA, meiosis-specific; dmc1 mutants are defective in reciprocal recombination and accumulate DSBs
11 ETEKRCKQKEQRY (SEQ ID NO:326)	PMS1		904 aa, 103 kDa; mismatch-repair protein; MutL (Salmonella) and HexB (Streptococcus) homolog
None 1 MDLRVGRKFRIGRKIG (SEQ ID NO:327) 139 GRRGX$_8$GLSKKYRDFNT HRHIP (Bipartite weak NLS) (SEQ ID NO:328)	HRR25	Hhp1, Hhp1 (S pom) CKI (mamm	Mutations in HRR25 Ser/Thr protein kinase cause defects in DNA repair and retardation in cell cycling
96 HELTKRSSRRVETEK (SEQ ID NO:329)	YKL510		383 aa; structure-specific endonuclease; two domains of about 100 aa with sequence similarity to N- and C-terminal regions of RAD2.
200 MLAMARRKKKMSAK (SEQ ID NO:330) 617 EHYKVKHTEK (weak NLS) (SEQ ID NO:331) 670 LHPEKKRSISE (weak NLS) (SEQ ID NO:332)	MOT1		Modifier of transcription 1; 1867 aa; DNA helicase of *S. cerevisiae* required for viability; increases gene expression of several., but not all, pheromone-responsive genes in the absence of STE12; the 1257 to 1825 aa domain (568 aa residues) has homology to SNF2 and RAD54
S. POMBE			
60 SSIDEx$_5$SIKRKRRI (SEQ ID NO:333)	Swi4	Duc-1 Rep-3	113 kDa; KCII sites are upstream of NLS like in SV40 large T; the homologous prokaryotic MutS and HexA lack NLS
96 GELAKRVARHQKARE (weak NLS) (SEQ ID NO:334) 362 GSAKRKRDS (SEQ ID NO:335) 372 KGGESKKKR (SEQ ID NO:336)	Rad2		380 aa
None	Rad9	-	427 aa; no homology to other DNA repair proteins; rad9 fission yeast mutants are sensitive to both UV and ionizing radiation; may be involved in recombination-repair.
None 681 DKRYGRSDKRTKLPK (SEQ ID NO:337)	Rhp3 or rad15	ERCC2 RAD3	772 aa; DNA helicase; 65% identity to RAD3 and 55% identity to ERCC2; essential for viability

Putative NLS	Gene product	Equivalent protein in other species	Features
464 PPSKRRRVRGG (SEQ ID NO:338)	Rad16	RAD1	Function in repair of UV damage for both cyclobutane dimer and (6-4) photoproduct lesions; Rad16 interacts with Swi10.
431 DFKQAILRKRKNESPE EVEP (SEQ ID NO:339)	Rad21		628 aa, 67.8 kDa, acidic protein; a single base substitution in mutant rad21-45, changing an Ile into a Thr, is responsible for the low efficiency in repair of DSBs after g-radiation although capable of arresting at G2.
490 DKKAKKG (SEQ ID NO:340)	Rad22	RAD52	496 aa; functions in recombination-repair and mating-type switching.
394 DVVQFYLKKKYTRSKRN DG (weak because of Y) (SEQ ID NO:341) 575 PSPALLKKTNKRRELP (SEQ ID NO:342)	Rad32	MRE11 (S cer)	648 aa; meiotic recombination protein; rad32 mutants are sensitive to g- and UV radiation; functions in the same pathway with Rhp51 (RAD51).
	Rad51		recombination-repair
GLAKKYRDHKTHLHIP (weak NLS because of Y and H) (SEQ ID NO:343)	Hhp1	CKI (mamm) HRR25 (S cer)	Ser/Thr protein kinase; mutation in this gene causes repair defects
None GLAKKYRD[F]KTHVHIP (H in Hhp1 is replaced by F in Hhp2) (SEQ ID NO:344)	Hhp2	CKI (mamm) HRR25 (S cer)	Ser/Thr protein kinase; mutation in this gene causes repair defects

Table 9. NLS in Transcription factors

NLS and Flanks	Protein factor and features
highly basic	
HR4QRTRK7R (SEQ ID NO:345) LRRKSRP (SEQ ID NO:346) SRRTKRRQ (SEQ ID NO:347)	Human GCF (GC-factor)
GRKRKKRT (SEQ ID NO:348)	Oct-6 protein transcription factor from mouse cells
GRRRKKRT (SEQ ID NO:349)	Mouse Oct-2 protein transcription factors (Oct-2.1 for Oct-2.6 isoforms)
ARKRKRT (SEQ ID NO:350) NRRQKGKRS (SEQ ID NO:351)	Oct-3 from mouse P19 embryonal carcinoma cells
ECRRKKKE (SEQ ID NO:352)	Human ATF-1. In basic region/leucine zipper.
ERKKRRRE (SEQ ID NO:353) AKCRNKKKEKT (SEQ ID NO:354)	Human ATF-3 (in basic region that binds DNA)
SKKKIRL (SEQ ID NO:355) QKGNRKKM (SEQ ID NO:356) VKKVKKKL (SEQ ID NO:357)	Mouse Pu.1 (Friend erythroleukemia cells). Related to ets oncogene
VKRKKI (SEQ ID NO:358) CRNRYRKLE (SEQ ID NO:359) IRKRRKMK (SEQ ID NO:360) PKKKRLRL (SEQ ID NO:361)	Human PRDII-BF1 that binds to IFN-β gene promoter. (The largest DNA-binding protein known, of 298 kD).
GKKKKRKREKL (within the HMG-box) (SEQ ID NO:362)	Murine LEF-1 (397 aa). Lymphoid-specific with an HMG1-like box. NLS is identical to that of human TCF-1α.
GKKKKRKREKL (within the HMG-box) (SEQ ID NO:363)	Human TCF-1α (399 aa) (T cell-specific transcription factor that activates the T cell receptor Cα). Contains an HMG box. NLS core is identical to that of murine LEF-1.
GKKKRRSREKH (within the HMG-box) (SEQ ID NO:364) PKKCRARF (SEQ ID NO:365)	Human TCF-1 (uniquely T cell-specific). HMG box containing.
FKQRRIKL (SEQ ID NO:366) NRRRKKRT (SEQ ID NO:367) NRRQKEKRI (SEQ ID NO:368)	*Xenopus* laevis Oct-1(within POU-domain)
DKRSRKRKRSK (SEQ ID NO:369) RLRIDRKRN (SEQ ID NO:370) AKRSRRS (SEQ ID NO:371)	*Drosophila* Suvar (3) 7 gene product involved in position-effect variegation (932 aas). Five widely spaced zinc-fingers could help condensation of the chromatin fiber.

NLS and Flanks	Protein factor and features
IRKRRKMKSVGD2E2 (SEQ ID NO:372) (not suggested as NLS by the authors; between the 1st and 2nd zinc finger) PPKKKRLRLAE (suggested as NLS by the authors; just before 2nd zinc finger) (SEQ ID NO:373) CRNRYRKLE (within 1st zinc finger) (SEQ ID NO:374)	Human MBP-1 (class I MHC enhancer binding protein 1) mw 200 kD. Induced by phorbol esters and mitogens in Jurkat T cells.
PRRKRRV (SEQ ID NO:375) HRYKMKRQ (SEQ ID NO:376)	rat TTF-1 (thyroid nuclear factor that binds to the promoter of thyroid-specific genes). An homeodomain protein.
DGKRKRKN (SEQ ID NO:377) DDSKRVAKRKL (SEQ ID NO:378) NRERRRKEE (SEQ ID NO:379) WKQRRKF (SEQ ID NO:380)	Human thyroid hormone receptor α (c-erbA-1 gene). Belongs to the family of cytoplasmic proteins that are receptors of hydrophobic ligands such as steroids, vitD, retinoic acid, thyroid hormones. The ligand binding may expose the NLS for nuclear import of the receptor-ligand complex.
NRRKRKRS (SEQ ID NO:381) PKKKKL (SEQ ID NO:382)	*Drosophila* gcl (germ cell-less) gene product (569 aa, 65 kD), located in nuclei, required for germ line formation.
ARRKRRRL (SEQ ID NO:383) LKFKKVRD (SEQ ID NO:384) FKKFRKF (SEQ ID NO:385) GKQKRRF (SEQ ID NO:386) ERLKRDKEKREKE (SEQ ID NO:387) TRGRPKKVKE (SEQ ID NO:388) SKKRGRRRKKT (SEQ ID NO:389) TRRQKRAKV (SEQ ID NO:390) SRKSKKRLRA (SEQ ID NO:391)	*C. elegans* Sdc-3 protein (sex-determining protein) (2,150 aas). A zinc finger protein.
LKKIRRKIKNKI (SEQ ID NO:392) ESRRKKKE (SEQ ID NO:393)	*Drosophila* BBF-2 (related to CREB/ATF)
Group θθθθ	
DRNKKKKE (SEQ ID NO:394) ARRRRP (SEQ ID NO:395)	*Xenopus* RAR (retinoic acid receptor)
GRRRRA (SEQ ID NO:396) DEKRRKV (SEQ ID NO:397) CRQKRKV (SEQ ID NO:398)	Human ATF-2 (the 2nd and 3rd NLS are in basic region that binds DNA)
ERKRRD (SEQ ID NO:399) SRKKLRME (SEQ ID NO:400)	Myn (murine homolog of Max). Forms a specific DNA-binding complex with c-Myc oncoprotein through a helix-loop-helix/leucine zipper.
EEKRKRTYE (SEQ ID NO:401)	human NFκB p65 (550 aa). Not binding DNA; complexed with p50 that binds DNA. NFκB p50 also contains a NLS (Table 3b).

NLS and Flanks	Protein factor and features
GRRRRA (SEQ ID NO:402) DEKRRKF (SEQ ID NO:403) SRCRQKRKV (SEQ ID NO:404)	Human HB16, a cAMP response element-binding protein
SKKKKTKV (SEQ ID NO:405) NRPDKKKI (SEQ ID NO:406) QRRKKP (SEQ ID NO:407) QKKRRFKT (SEQ ID NO:408)	Human TFIIE-β (general transcription initiation protein factor; forms tetramer $\alpha_2\beta_2$ with TFIIE-α)
SRKRKM (SEQ ID NO:409)	Human kup transcriptional activator (433 aas). Two distantly spaced zinc fingers. Expressed in hematopoietic cells and testis.
ERKRLRNRLA (SEQ ID NO:410) ATKCRKRKL (SEQ ID NO:411) (19 aa stretch)	Mouse Jun-B homologue to avian sarcoma virus 17 oncogene v-jun product. One region is similar to yeast GCN4 and to Fos.
DKRx_6ERKRRD (N-terminus) (SEQ ID NO:412) QSRKKLRME (C-terminus) (SEQ ID NO:413)	Max (specifically associates with c-Myc, N-Myc, L-Myc). The Max-Myc complex binds to DNA; neither Max nor Myc alone exhibit appreciable DNA binding.
DKEKKIKLEEDE (within an acidic region) (SEQ ID NO:414) IKKAKKV (SEQ ID NO:415) TRRKKN (SEQ ID NO:416)	Chicken VBP (vitellogenin gene-binding protein). Leucine zipper. Related to rat DBP.
TRDDKRRA (SEQ ID NO:417) EVERRRRDK (SEQ ID NO:418)	*Xenopus* borealis B1 factor. Closely related to the mammalian USF. Binds to CACGTG in TFIIIA promoter to developmentally regulate its expression.
TRDEKRRA (SEQ ID NO:419) EVERRRRDK (SEQ ID NO:420)	Human USF (upstream stimulatory factor) activating the major late adenovirus promoter
YRRYPRRRG (SEQ ID NO:421) QRRPYRRRRF (SEQ ID NO:422) YRPRFRRG (SEQ ID NO:423) QRRYRRN (SEQ ID NO:424) YRRRRP (SEQ ID NO:425)	YB-1, a protein that binds to the MHC class II Y box. YB-1 is a negative regulator.
AKERQKKD (SEQ ID NO:426) ERRRRF (SEQ ID NO:427)	Human TFEB Binds to IgH enhancer.
LKERQKKD (SEQ ID NO:428) IERRRRFN (SEQ ID NO:429) YFRRRRLEKD (SEQ ID NO:430)	Human TFE3 (536 aa). Binds to μE3 enhancer of IgH genes.
KTVALKRRKASSRL (SEQ ID NO:431)	Human Drl (176 aa, 19 kD). Interacts with TBP (TATA-binding protein) thus inhibiting association of TFIIA and/or TFIIB with TBP. TBP-Drl association is affected by Drl phosphorylation to repress activated and basal transcription.
1 LRRRGRQTY (SEQ ID NO:432) 27 LTRRRRIEM (SEQ ID NO:433) 51 QNRRMKLKKEI (SEQ ID NO:434)	*Drosophila* ultrabithorax protein (from the conserved 61 amino acid homeodomain segment only). Conserved in the antenappedia homeodomain protein.

NLS and Flanks	Protein factor and features
SNRRRPDHR (SEQ ID NO:435) VYRGRRRVRRE (SEQ ID NO:436) $P_7AP_2RRRRSADNKD_2$ (SEQ ID NO:437) PKKPRHQF (SEQ ID NO:438)	*C. elegans* sex-determining Tra-1 protein. Zinc finger. Peaks in the second larval stage.
EKRKKERN (SEQ ID NO:439) LLRRLKKEVE (SEQ ID NO:440) $EPLGRIRQKKRVY_2D_2$ (SEQ ID NO:441) (EDAIKKRREARERRRLRQ) (SEQ ID NO:442) DKETTASRSKRRSSRKKRT (SEQ ID NO:443) ESKKKKPKL (SEQ ID NO:444) KKTAAKKTKTKS (SEQ ID NO:445)	Yeast NPS1 transcription protein factor (1359 aa) involved in cell growth control at G2 phase. Has a catalytic domain of protein kinases.
QRKRQKL (SEQ ID NO:446) KAKKQK (SEQ ID NO:447) LRRKRQK (SEQ ID NO:448)	Human 243 transcriptional activator (968 aas), induced by mitogens in T cells. N-terminal half is homologous to oncoprotein Rel and *Drosophila* Dorsal protein involved in development. The C-terminal half contains repeats found in proteins involved in cell-cycle control of yeast and tissue differentiation in *Drosophila*.
RDIRRRGKNKV (SEQ ID NO:449) QNCRKRKLE (SEQ ID NO:450)	Mouse NF-E2 (45 kD), an erythroid transcription factor from mouse erythroleukemia (MEL) cells. Involved in globin gene regulation. Binds to AP-1-like sites. Homology to Jun B, GCN4, Fos, ATF1 and CREB in basic region/leucine zipper (see Fig. 2).
Group θθθxθθ	
DKIRRKN (SEQ ID NO:451) ARKTKKKI (SEQ ID NO:452)	Human glucocorticoid receptor
473 DKIRRKNCP (SEQ ID NO:453) EARKTKKKIKGIQ (SEQ ID NO:454)	Mouse and human GR (glucocorticoid recptor)
Group θθθxθ	
YRVRRERN (SEQ ID NO:455) VRKSRDKA (SEQ ID NO:456) DRLRKRVE (SEQ ID NO:457)	C/EBP (CCAAT/enhancer binding protein). Functions in liver-specific gene expression.
DKIRRKN (SEQ ID NO:458) ARKSKKL (SEQ ID NO:459)	Human mineralocorticoid receptor
DKIRRKN (SEQ ID NO:460) GRKFKKF (SEQ ID NO:461)	Human PR (progesterone receptor)
EEVQRKRQKLMP (SEQ ID NO:462)	Human and mouse NFκB 105 kD precursor of p50 (968 aas) (first R is at 361 position).
EEVQRKRQKL (SEQ ID NO:463)	Human NF-κB p50 (DNA-binding subunit). Identical to protein KBF1, homologous to rel oncogene product. NF-κB p65 also contains a NLS (Table 3a).
GKTRTRKQ (SEQ ID NO:464) ARRKSRD (SEQ ID NO:465)	Human TEF-1 (SV40 transcriptional enhancer factor 1). 426 aa.

NLS and Flanks	Protein factor and features
QRKERKSKS (SEQ ID NO:466) TKSKTKRKL (SEQ ID NO:467)	Rat, mouse, human IRF-1 (interferon regulatory factor-1). Induced in lymphoma T cells by the pituitary peptide hormone prolactin. Regulates the growth-inhibitory interferon genes.
GKCKKKN (SEQ ID NO:468)	Ehrlich ascites S-II transcription factor. A general factor that acts at the elongation step.
ERSKKRSRE (SEQ ID NO:469) ERELKREKRKQ (SEQ ID NO:470) ARRSRLRKQ (SEQ ID NO:471)	Tobacco TAF-1 transcriptional activator
YKLDHMRRRIETDE (SEQ ID NO:472)	*Drosophila* TFIIEα (433 aa), a general transcription factor for RNA polymerase II. Composed of subunits α and β.
DKNRRKS (SEQ ID NO:473) IRKDRRG (SEQ ID NO:474) IKRSKKN (SEQ ID NO:475)	Human ER (estrogen receptor); 595 aa.
EQRRHRIE (SEQ ID NO:476) TTRAEKKRLL (SEQ ID NO:477) IDKKRSKEAKE (SEQ ID NO:478)	Yeast ADA2 (434 aa), a potential transcriptional adaptor required for the function of certain acidic activation domains.
EAALRRKIRTISK (SEQ ID NO:479)	Yeast GCN5 gene product (439 aa), required for the function of GCN4 transcriptional activator and for the activity of the HAP2-3-4 complex.
Group θθxθθ	
NKKMRRNRF (SEQ ID NO:480) NRRKx4RQK (SEQ ID NO:481)	Mouse LFB3
TKKGRRNRF (SEQ ID NO:482) NRRKx4RHK (SEQ ID NO:483)	Mouse LFB1
NKKMRRNRFK (SEQ ID NO:484)	rat vHNF1-A
NKKMRRNR (SEQ ID NO:485)	murine HNF-1β
TKKGRRNRF (SEQ ID NO:486)	mouse HNF-1
NKKMRRNRF (SEQ ID NO:487)	human vHNF1
TKKGRRNRF (SEQ ID NO:488)	rat liver HNF1
LRRQKRFK (SEQ ID NO:489) QQH3SH4Q (SEQ ID NO:490)	rat HNF-3β
LRRQKRFK (SEQ ID NO:491)	rat HNF-3γ
LRRQKRFK (SEQ ID NO:492)	rat HNF-3α
LKEKERKA (SEQ ID NO:493) MKKARKV (SEQ ID NO:494)	rat DBP a protein factor that binds to the D site of the albumin gene promoter
PRRERRY (SEQ ID NO:495)	rat AT-BP1. Highly acidic domain. Two zinc fingers. Binds to the B-domain of α_1-antitrypsin gene promoter and to the NF-κB site in the MHC gene enhancer.
DRRVRKGKV (SEQ ID NO:496)	A 19 kD *Drosophila melanogaster* nonhistone associated with heterochromatin.

NLS and Flanks	Protein factor and features
SKHGRRARRLDP (SEQ ID NO:497)	murine EBF (early B-cell factor) of 591 aa. Regulates the pre-B and B lymphocyte-specific mb-1 gene. Expressed in pre-B and B-cell lines but not in plasmocytomas, T-cell and nonlymphoid cell lines.
GRRTRRE (SEQ ID NO:498)	human Sp1
DEQKRAEKKAKE (SEQ ID NO:499) IRRIHKVIRP (SEQ ID NO:500) LLRRLKKDVE (SEQ ID NO:501)	yeast SNF2, a transcriptional regulator of many genes.
Group θxθθxθ	
AKAKAKKA (SEQ ID NO:502) YKMRRERN (SEQ ID NO:503) VRKSRDKA (SEQ ID NO:504)	mouse AGP/EBP (87% similarity to C/EBP), ubiquitously expressed
AKAKAKKA (SEQ ID NO:505) YKMRRERN (SEQ ID NO:506) VRKSRDKA (SEQ ID NO:507)	rat LAP, a 32-kD liver-enriched transcriptional activator, also present in lung, with 71% sequence similarity to C/EBP. Leucine zipper. Accumulates to maximal levels around birth.
YRQRRER (SEQ ID NO:508) VKKSRLKSKQK (SEQ ID NO:509)	Ig/EBP-1 (immunoglobulin gene enhancer-binding protein). Forms heterodimers with C/EBP.
EDPEKEKRIKELE (SEQ ID NO:510) MRRKV (SEQ ID NO:511)	mouse c-Myb
DYYKVKRPKTD (SEQ ID NO:512) GRARGRRHQ (SEQ ID NO:513) FRYRKIKDIY (SEQ ID NO:514)	*Drosophila* eyes absent protein (760 aa), a nuclear protein that functions in early development to prevent programmed cell death and to allow the event that generate the eye to proceed. Mutations cause programmed cell death of eye progenitor cells.
Group θxθxθθ	
AKAKAKKA (SEQ ID NO:515)	rat IL-6DBP interacting with interleukin-6 responsive elements. Has a leucine zipper domain.
DKRQRNRC (SEQ ID NO:516) FkrtirkD	mouse H-2RIIBP (MHC class I genes H-2 region II binding protein). Member of the nuclear hormone receptor superfamily.
FkrtirkD DKRQRNRC (SEQ ID NO:517)	chicken RXR, related to RAR (retinoic acid receptor), a nuclear protein factor from the thyroid/steroid hormone receptor family
VKSKAKKT (SEQ ID NO:518) YKIRRERN (SEQ ID NO:519) VRKSRDKA (SEQ ID NO:520)	human NF-IL6 (345 aa). Specifically binds to IL1-responsive element in the IL-6 gene. Leucine zipper. Homology to C/EBP.
QKKNRNKC (SEQ ID NO:521)	mouse PPAR (peroxisome proliferator activated receptor)
Group θθθxxθθ	
EQIRKLVKKHG (SEQ ID NO:522)	yeast RAP1 It binds regulatory sites at yeast mating type silencers.
FRRSMKRKA (SEQ ID NO:523)	human vitamin D receptor (427 aa)
Group θθxxθθ	

NLS and Flanks	Protein factor and features
LKRHQRRH (SEQ ID NO:524)	mouse WT1 (the murine homolog of human Wilms' tumor predisposition gene WT1)
LKRHQRRH (SEQ ID NO:525)	human WT33 (Wilms' tumor predisposition)
Group θθθxxθ	
LKESKRKYDE (SEQ ID NO:526)	yeast SWI3 99 kD, highly acidic protein. Global transcription activator.
EVLKVQKRRIYD (SEQ ID NO:527)	human RBAP-1 (retinoblastoma-associated protein 1) factor (412 aa). A protein that binds to the pocket (functional domain) of the retinoblastoma (RB) protein involved in suppression of cell growth (tumor suppressor). The transcription factor E2F, implicated in cell growth, binds to the same pocket of RB.

Table 10. NLS in other nuclear proteins

Putative NLS	Protein
YKSKKKA (SEQ ID NO:528) TKKLPRKT (SEQ ID NO:529)	Yeast L3
TRKKGGRRGRRL (SEQ ID NO:530) C-terminus	Yeast 59 ribosomal protein
ARATRRKRCKG (SEQ ID NO:531)	Yeast L16 ribosomal protein
GKGKYRNRRW (SEQ ID NO:532)	yeast L2 ribosomal protein (homologous to *Xenopus* L1). Encoded by intronless genes.
GKGKMRNRRRIQRRG (SEQ ID NO:533) NKKVKRRELKKN (SEQ ID NO:534) AKTARRKA (SEQ ID NO:535) IKAKEKKP (SEQ ID NO:536) GKPKAKKP (SEQ ID NO:537) AKAKKRQ (SEQ ID NO:538)	*Xenopus* laevis L1 ribosomal protein (homologous to yeast L2) Encoded by intronless genes.
ERKRKS (SEQ ID NO:539) GKRPRTKA (SEQ ID NO:540) HKRRRI (SEQ ID NO:541) LKKQRTKKNKE (SEQ ID NO:542)	human S6 ribosomal protein (homologous to yeast S10)
PKMRRRTYR (SEQ ID NO:543) KKKISQKKLKK (SEQ ID NO:544)	Rat L17 ribosomal protein (184 aas)
YMRRRTYRA (SEQ ID NO:545) EVKKVSKKKL (SEQ ID NO:546)	Podocoryne carnea (hydrozoan, Coelenteratum) L17 ribosomal protein (184 aas) highly homologous to rat L17.
ERNRKDKDAKFR (SEQ ID NO:547)	human, rat ribosomal S13 protein
ERKRKS (SEQ ID NO:548) QRLQRKRH (SEQ ID NO:549) IRKRRA (SEQ ID NO:550)	yeast S10 ribosomal protein (homologous to human S6)
GRRRKKHRSRSRSRERRSRSRDRGRG$_{12}$GRER DRRRSRDRER (SEQ ID NO:551)	35 kD subunit of U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), an essential mammalian splicing factor. U2AF^{35} interacts with the 65 kD subunit (U2AF^{65}). Both proteins are concentrated in a small number of subnuclear organelles, the coiled bodies.
EFEDPRD (SEQ ID NO:552) ETREERME (SEQ ID NO:553) EAGDAPPDP (SEQ ID NO:554) EERMERKRREK (SEQ ID NO:555) HRDRDRDRERERRESRERDKERERRRSRSRD RRRRSRSRDKEERRRSRERSKDKDRDRKRRS SRSRERARRERERKEE (SEQ ID NO:556) RDRDRERRRSHRSERERRRDRDRDRDRDREH KRGER (SEQ ID NO:557)	human UsnRNP-associated 70 k protein (437 aas) that is phosphorylated at Arg/Ser-rich domains; involved in splicing
QKRNNKKSKKKRCAE (SEQ ID NO:558) EKLRKLKI (near C-terminus) (SEQ ID NO:559)	yeast TRM1 enzyme for the N^2,N^2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNAs. TRM1 is both nuclear and mitochondrial. The first motif is within a region (70-213 aa segment) known to cause nuclear localization of β-galactosidase.
NKRKRV (SEQ ID NO:560) SLKNRSNRKRE (SEQ ID NO:561) EPKRKRRLP (SEQ ID NO:562) ARMRHSKR (C-terminus) (SEQ ID NO:563)	Yeast nucleoporin NUP1 (1076 aa, 113 kD); an integral component of the pore complex. Involved in both binding and translocation steps of nuclear import.

Putative NLS	Protein
KAEKEx3KVD2E2 (SEQ ID NO:564) Kx3Kx5Kx3R (SEQ ID NO:565)	Chicken, *Xenopus* No 38 nucleolar (38 kD); involved in intranuclear packaging of preribosomal particles. Shuttles between nucleus and cytoplasm.
KTEREAEKALEEKx7R (SEQ ID NO:566) Kx5Kx7Kx4RX3EDTTEETLR (SEQ ID NO:567) RG2RG2RG3RG2FG2RG3RGFG2RG3FRG2RG4 DHKPQGKKIKFE (SEQ ID NO:568) (C-terminus)	Chicken, hamster nucleolin (92 kD). Binds preribosomal RNA. Shuttles between nucleus and cytoplasm.
WYKHFKKTKD (SEQ ID NO:569)	human SATB1 (763 aa) which binds selectively to AT-rich MARs with mixed A, T, C on one strand excluding G. Binds to minor groove with little contact with bases.
QKKKQMKAD (SEQ ID NO:570) (KKEKKE)5 (SEQ ID NO:571) KKEKKRKSED (SEQ ID NO:572) EEKKSKKSKK (SEQ ID NO:573)	yeast CBF5p, a centromere-binding protein (55kDa, 483aa). The KKE repeat at its C-terminus occurs in microtubule-binding domains; yeast cells containing only three copies of the KKE repeat of CBF5p delay at G2/M; depletion of CBE5p arrests cells at G1/S.
TKKKSFKL (SEQ ID NO:574)	yeast CCE1, a cruciform cutting endonuclease
KSERERMLRESLKEERRRF (SEQ ID NO:575)	rat nucleoporin 155 or Nup155 (1390 aas, 155 kDa), a protein of the nuclear pore complex; contains 46 consensus sites for various kinases; associated with both the nucleoplasmic and the cytoplasmic region of pores.
PKKGSKKA (SEQ ID NO:576) DGKKRKRSRKES (SEQ ID NO:577)	human H2B variant differentially expressed during the cell cycle
GAKRHRKVLRD (SEQ ID NO:578) 14-24 PAIRRLARRG (SEQ ID NO:579) 32-41 EHARRKT (SEQ ID NO:580) 74-80	Calf thymus histone H4 (102 aa)
ARRIRGERA 127-135 (SEQ ID NO:581)	Calf thymus H3 (135 aa)
GSHHKAKGK 121-129 (SEQ ID NO:582)	Calf thymus H2A (129 aa)
RGKSGKARTKAKSRSSR 3-19 (SEQ ID NO:583)	Sea urchin *Psammechinus miliaris* H2A (123 aa)
PKKGSKKA 10-17 (SEQ ID NO:584) QKKDGKKRKRSRKES 22-36 (SEQ ID NO:585)	Calf thymus H2B (125 aa)
GGKKRHRKRKGSY (SEQ ID NO:586) 22-34	Sea urchin *Psammechinus miliaris* H2B (122 aa)
PRTDKKRRRKRKES 19-32 (SEQ ID NO:587)	Starfish H2B (121 aa)
PAKAPKKKA 12-20 (SEQ ID NO:588) EAKKPAKKA 104-112 (SEQ ID NO:589) AKKPKKV 128-134 (SEQ ID NO:590) AKKSPKKAKKP 142-152 (SEQ ID NO:591) PKKVKKP 183-189 (SEQ ID NO:592)	Trout testis H1 (194 aa)

Putative NLS	Protein
PRRKAKRA 30-37 (SEQ ID NO:593) PKKAKKT 119-125 (SEQ ID NO:594) AKAKKAKA 129-136 (SEQ ID NO:595) AKKARKAKA 139-147 (SEQ ID NO:596) AKKAKKPKKKA 171-181 (SEQ ID NO:597) AKKAKKPAKK 182-191 (SEQ ID NO:598) SPKKAKKP 192-199 (SEQ ID NO:599) AKKSPKKKKAKRS 200-212 (SEQ ID NO:600) PKKAKKA 213-219 (SEQ ID NO:601) AKKAKKS 227-233 (SEQ ID NO:602) PRKAGKRRSPKKARK 234-248 (SEQ ID NO:603)	Sea urchin *Parechinus angulosus* sperm H1 (248 aa)
ARRRKTA 1-7 (SEQ ID NO:604) IRKFIRKA 55-61 (SEQ ID NO:605) PKKKKA 83-88 (SEQ ID NO:606) AKKPKAKKVKKP 89-100 (SEQ ID NO:607) AKKKTNRARKPKTKKNR 104-120 (SEQ ID NO:608)	Annelid sperm H1a (119 aa)
PKRKVSS 1-7 (SEQ ID NO:609) EEPKRRSARLS 14-24 (SEQ ID NO:610)	Calf thymus HMG14 (100 aa)
PKRKAEGDAK 1-10 (SEQ ID NO:611) PKGKKGKA 52-59 (SEQ ID NO:612)	Calf thymus HMG17 (89aa; 9,247 D)
PKKPRGKM (SEQ ID NO:613) EHKKKHP (SEQ ID NO:614) ETKKKFKDP (SEQ ID NO:615) $EKSKKKK(E/D)_{41}$ (SEQ ID NO:616) $E_3G_2KKKKKFAK$ (SEQ ID NO:617)	Calf thymus HMG 1 (259 aa)
EHKKKHP (SEQ ID NO:618) PKGDKKGKKKDP (SEQ ID NO:619) $E_4G_3KKKKKFAK$ (SEQ ID NO:620)	Calf thymus HMG 2 (256 aa)
PKRKSATKGDEPARR 1-15 (SEQ ID NO:621) KPKKAAAPKKA 30-34 (SEQ ID NO:622)	Trout testis H6 (60 aa)

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Claims

What is claimed is:

1. A method for producing micelles with entrapped therapeutic agents, comprising:

a) combining an effective amount of a negatively charged therapeutic agent with an effective amount of a cationic lipid in a ratio where about 30% to about 90% the negatively charged atoms are neutralized by positive charges on lipid molecules to form an electrostatic micelle complex in about 20% to about 80% ethanol; and

b) combining the micelle complex of step a) with an effective amount of a fusogenic-karyophilic peptide conjugates in a ratio range of about 0.0 to about 0.3, thereby producing micelles with entrapped therapeutic agents.

2. The method of claim 1, wherein the negatively charged therapeutic agent is a therapeutic agent selected from the group consisting of a polynucleotide and a negatively charged drug.

3. The method of claim 2, wherein the polynucleotide is a DNA polynucleotide or an RNA polynucleotide.

4. The method of claim 2, wherein the polynucleotide is a DNA polynucleotide.

5. The method of claim 4, wherein the DNA polynucleotide comprises plasmid DNA.

6. The method of claim 1, further comprising combining an effective amount of an anionic lipid in step a).

7. The method of claim 6, wherein the anionic lipid is dipalmitoyl phosphatidyl glycerol (DDPG) or a derivative thereof.

8. The method of claim 4, further comprising combining an effective amount of a DNA condensing agent selected from the group consisting of spermine, spermidine, polylysine, polyarginine, polyhistidine, polyornithine and magnesium or a divalent metal ion.

9. The method of claim 5, wherein the plasmid DNA comprises a sequence encoding p53, HSV-tk, p21, Bax, Bad, IL-2, IL-12, GM-CSF, angiostatin, endostatin and oncostatin.

10. The method of claim 1, wherein the cationic lipids are selected from the group consisting of 3β-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol, dimethyldioctadecyl ammonium bromide (DDAB), N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide (DMRIE), 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP), dioctadecylamidoglycylspermine (DOGS), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), 1,2- dipalmitoyl-3-trimethylammonium propane (DPTAP), 1,2-disteroyl-3-trimethylammonium propane (DSTAP).

11. The method of claim 10, wherein the cationic lipids are combined with the fusogenic lipid DOPE in a molar ratio from about 1:1 to about 2:1.

12. The method of claim 11, wherein the cationic lipids are combined with the fusogenic lipid DOPE in a molar ratio of 1:1.

13. The method of claim 1, wherein the fusogenic-karyophilic peptide is an NLS peptide.

14. The method of claim 13, wherein the NLS peptide is a peptide selected from the group consisting of Seq. ID Nos. 20 –622.

15. The method of claim 1, wherein the fusogenic-karyophilic peptide conjugate is a sole fusogenic peptide.

16. The method of claim 1, wherein the NLS peptide component of the fusogenic-karyophilic peptide conjugate is an NLS peptide selected from the group consisting of Seq. ID Nos. 20-622.

17. The method of claim 1, wherein the fusogenic/NLS peptide conjugates comprise amino acid sequences selected from the group consisting of **(KAWLKAF)$_3$** (SEQ ID NO:1), GLF**KAAAKLLKSLWKLLLKA** (SEQ ID NO:2), LLL**KAFAKLLKSLWKLLLKA** (SEQ ID NO:3) as well as all derivatives of the prototype (Hydrophobic$_3$Karyophilic$_1$Hydrophobic$_2$Karyophilic$_1$)$_{2\text{-}3}$ where Hydrophobic is any of the A, I, L, V, P, G, W, F and Karyophilic is any of the K, R, or H, containing a positively-charged residue every 3rd or 4th amino acid, that form alpha helices and direct a net positive charge to the same direction of the helix.

18. The method of claim 1, wherein the fusogenic/NLS peptide conjugate comprise an amino acid sequence selected from the group consisting of GLFKAIAGFI**KNGWKGMIDGGGYC** (SEQ ID NO:4) from influenza virus hemagglutinin HA-2 and **YGRKKRRQRRR** (SEQ ID NO:5) from TAT of HIV.

19. The method of claim 1, wherein the fusogenic/NLS peptide conjugate comprise an amino acid sequence selected from the group consisting of MSGTFGGILAGLIGLL**(K/R/H)$_{1\text{-}6}$** (SEQ ID NO:6), derived from the N-terminal region of the S protein of duck hepatitis B virus but with the addition of one to six positively-charged lysine, arginine or histidine residues, and combinations of these, GAAIGLAWIPYFGPAA (SEQ ID NO:7) derived from the fusogenic peptide of the Ebola virus transmembrane protein; residues 53-70 (C-terminal helix) of apolipoprotein (apo) AII peptide, the 23-residue fusogenic N-terminal peptide of HIV-1 transmembrane glycoprotein gp41, the 29-42-residue fragment from

Alzheimer's beta-amyloid peptide, the fusion peptide and N-terminal heptad repeat of Sendai virus, the 56-68 helical segment of lecithin cholesterol acyltransferase.

20. The method of any of claim 13 to 19, wherein the NLS peptide component in fusogenic/NLS peptide conjugates are synthetic peptides containing the above said NLS but further modified by additional K, R, H residues at the central part of the peptide or with P or G at the N- or C-terminus.

21. The method of claim 13, wherein the fusogenic peptide/NLS peptide conjugates are linked to each other with a short amino acid stretch representing an endogenous protease cleavage site.

22. The method of claim 1, wherein the structure of the preferred prototype fusogenic/NLS peptide conjugate used in this invention is: P**KKRR**GPSP$(L/A/I)_{12\text{-}20}$ (SEQ ID NO:8) where $(L/A/I)_{12\text{-}20}$ is a stretch of 12-20 hydrophobic amino acids containing A, L, I, Y, W, F and other hydrophobic amino acids.

23. The method of claim 1, wherein the fusogenic/NLS peptide conjugates are added to the mixture of DNA/cationic lipid and are incorporated into micelles.

24. The method of claim 1, further comprising combining an effective amount of an encapsulating lipid solution to step b).

25. The method of claim 24, wherein the encapsulating lipid is a lipid comprising cholesterol (40%), dioleoylphosphatidylethanolamine (DOPE) (20%), palmitoyloleoylphosphatidylcholine (POPC) (12%), hydrogenated soy phosphatidylcholine (HSPC) (10%), distearoylphosphatidylethanolamine (DSPE) (10%), sphingomyelin (SM) (5%), and derivatized vesicle-forming lipid M-PEG-DSPE (3%).

26. The method of claim 24, wherein the encapsulating lipid is a liposome.

27. The method of claim 26, wherein the liposomes comprises vesicle-forming lipids and between about 1 to about 7 mole percent of distearoylphosphatidyl ethanolamine (DSPE) derivatized with an effective amount of polyethyleneglycol.

28. The method of claim 27, wherein the liposomes have a selected average size of about 80 to about 160 nm.

29. The method of claim 27, wherein the polyethyleneglycol has a molecular weight from about 1,000 to about 5,000 daltons.

30. A micelle with an entrapped therapeutic agent produced by the method of claim 1.

31. A liposome encapsulated therapeutic agent produced by the method of claim 24.

32. The method of claim 31, wherein the therapeutic agent further comprises regulation by a liver, spleen or bone marrow regulatory DNA sequence.

33. The method of claim 32, wherein the regulatory DNA sequence is nuclear matrix DNA isolated from liver, spleen or bone marrow cells.

34. A method for delivering a therapeutic agent *in vivo*, comprising administration of an effective amount of the micelle of claim 30 to a subject.

35. The method of claim 34, wherein the therapeutic agent further comprises regulation by a tumor-specific regulatory DNA sequence.

36. The method of claim 35, wherein the tumor-specific regulatory sequence is nuclear matrix DNA isolated from specific tumor cells.

37. A method for delivering a therapeutic agent *in vivo*, comprising administration of an effective amount of the liposome encapsulated agent of claim 31 to the subject.

38. The method of claims 34 or 37, wherein the administration is intravenous administration or by injection.

39. A micelle with an entrapped DNA polynucleotide produced by the method of claim 9.

40. A method for reducing tumor size in a subject comprising administration of an effective amount of the micelle of claim 39 to the subject.

41. The method of claim 40, further comprising administration of an effective amount of a second therapeutic agent, wherein the agent is selected from the group consisting of ganciclovir, 5-fluorocytosine, an antisense oligonucleotides a ribozyme, and a triplex-forming oligonucleotide directed against genes that control the cell cycle or signaling pathways.

42. The method of claim 41, further comprising administration of an effective amount of a second therapeutic agent, wherein the second therapeutic agent is selected from the group consisting of adriamycin, angiostatin, azathioprine, bleomycin, busulfane, camptothecin, carboplatin, carmustine, chlorambucile, chlormethamine, chloroquinoxaline sulfonamide, cisplatin, cyclophosphamide, cycloplatam, cytarabine, dacarbazine, dactinomycin, daunorubicin, didox, doxorubicin, endostatin, enloplatin, estramustine, etoposide, extramustinephosphat, flucytosine, fluorodeoxyuridine, fluorouracil, gallium nitrate, hydroxyurea, idoxuridine, interferons, interleukins, leuprolide, lobaplatin, lomustine, mannomustine, mechlorethamine, mechlorethaminoxide, melphalan,

mercaptopurine, methotrexate, mithramycin, mitobronitole, mitomycin, mycophenolic acid, nocodazole, oncostatin, oxaliplatin, paclitaxel, pentamustine, platinum-triamine complex, plicamycin, prednisolone, prednisone, procarbazine, protein kinase C inhibitors, puromycine, semustine, signal transduction inhibitors, spiroplatin, streptozotocine, stromelysin inhibitors, taxol, tegafur, telomerase inhibitors, teniposide, thalidomide, thiamiprine, thioguanine, thiotepa, tiamiprine, tretamine, triaziquone, trifosfamide, tyrosine kinase inhibitors, uramustine, vidarabine, vinblastine, vinca alcaloids, vincristine, vindesine, vorozole, zeniplatin, zeniplatin, and zinostatin.

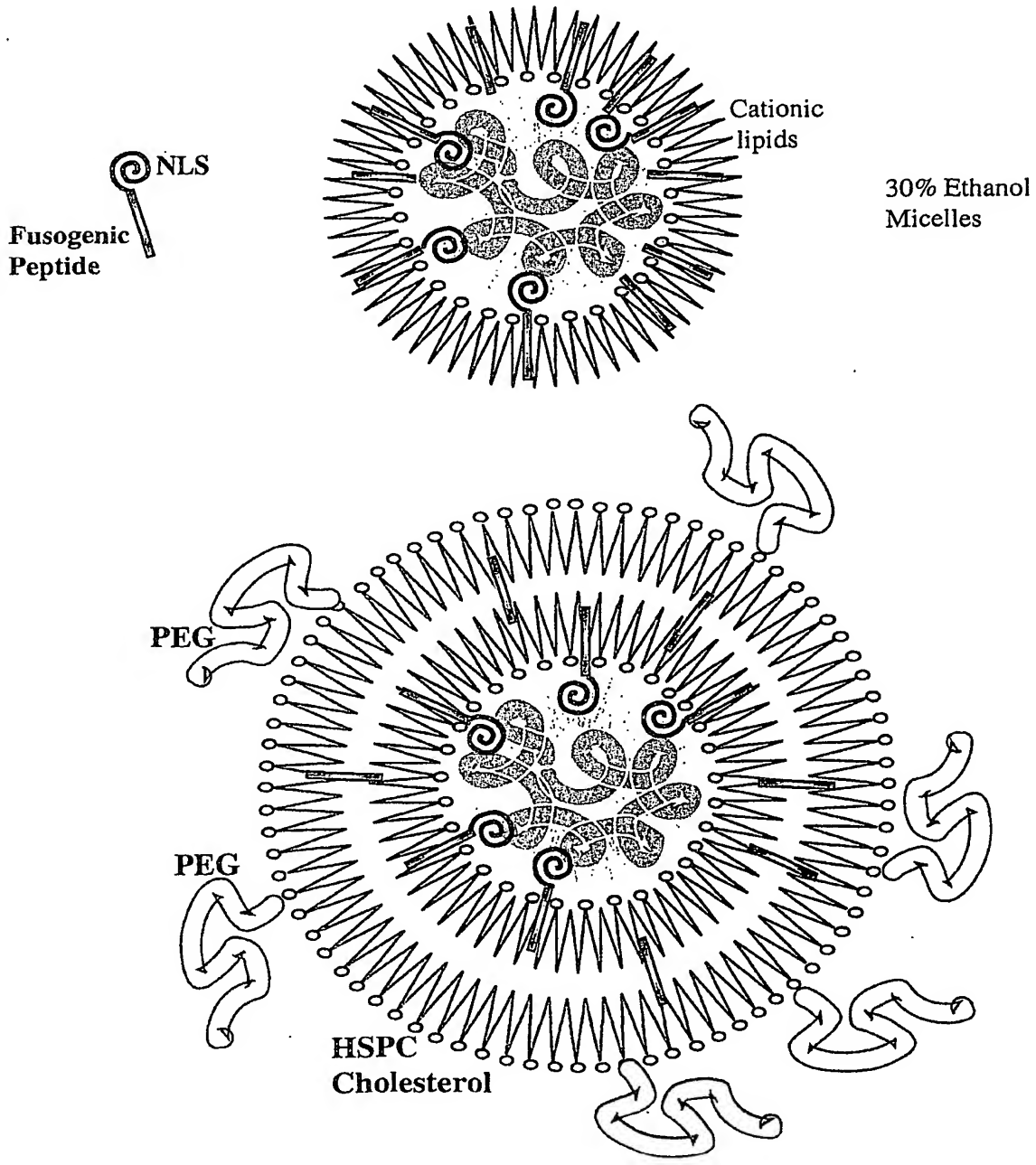
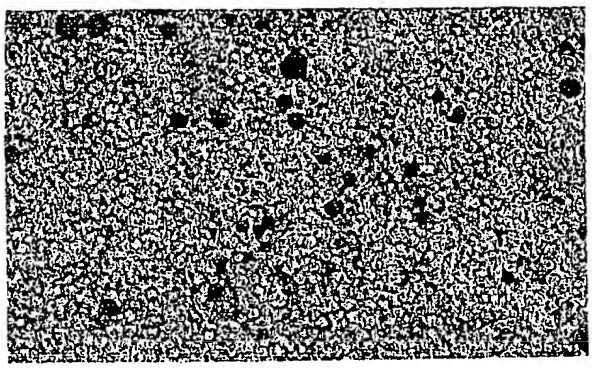
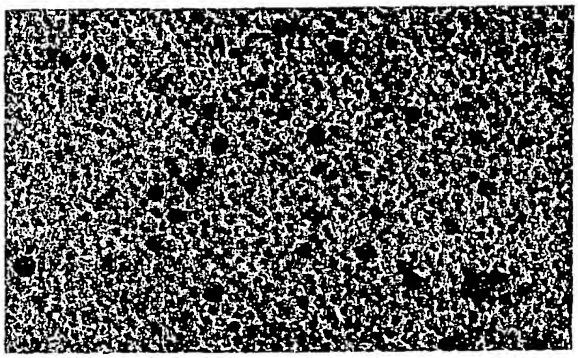

NLS
Fusogenic
Peptide
Cationic
lipids
30% Ethanol
Micelles
PEG
PEG
HSPC
Cholesterol

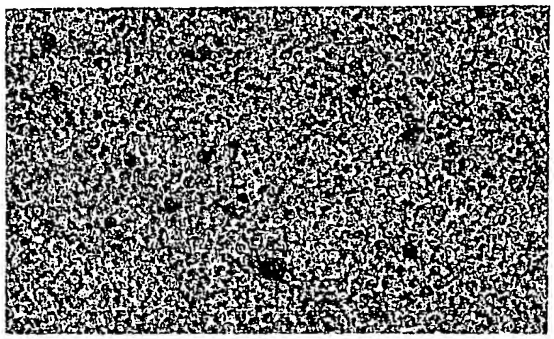
FIGURE 1



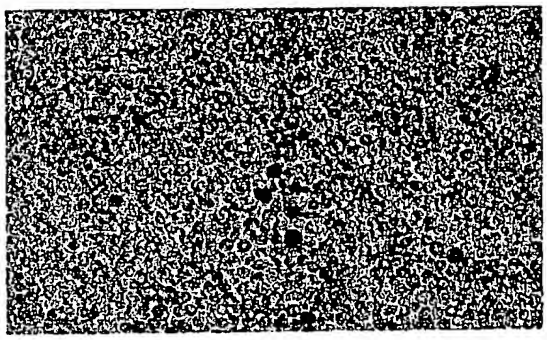
L2



L2+polyK



L2+ PolyChitosan



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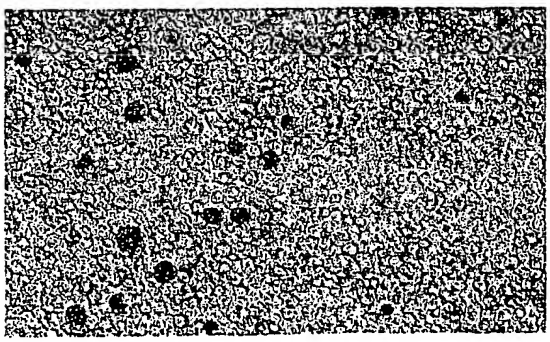
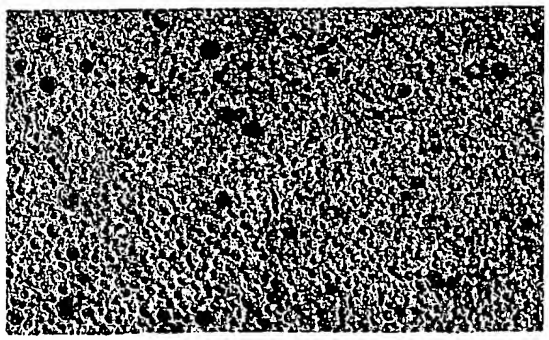
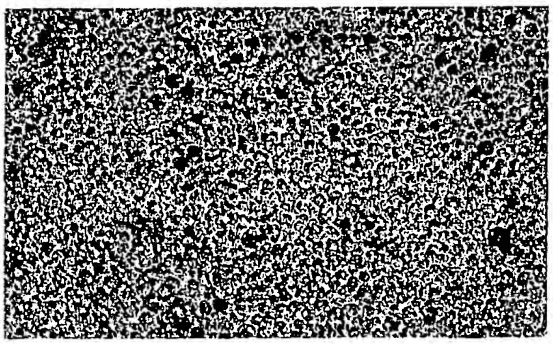


L2+PolyH

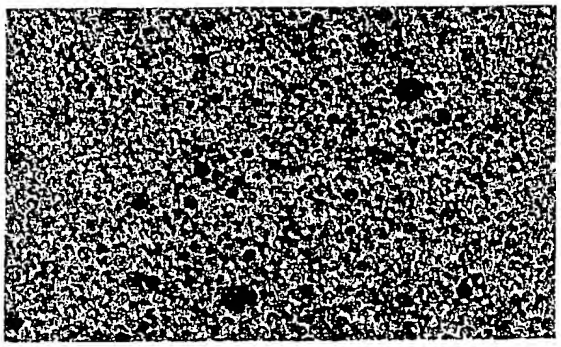


L2+polyOrnithine

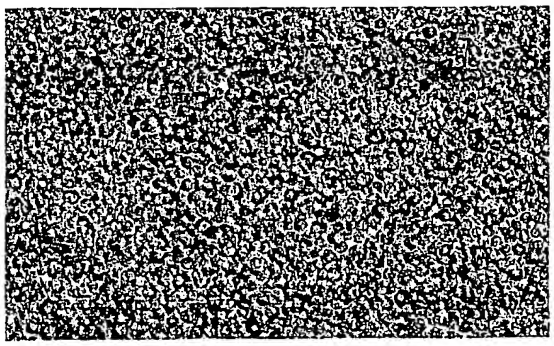
FIGURE 2



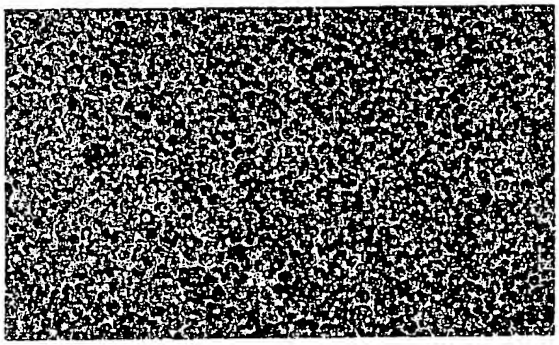
L2+polyR



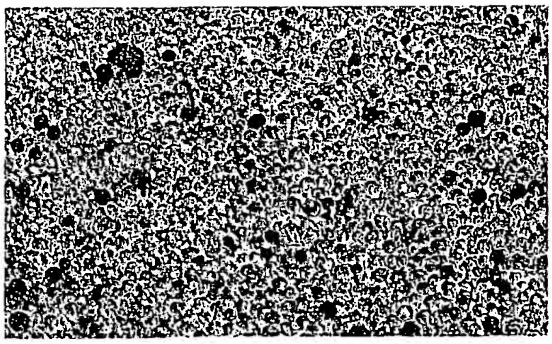
L5



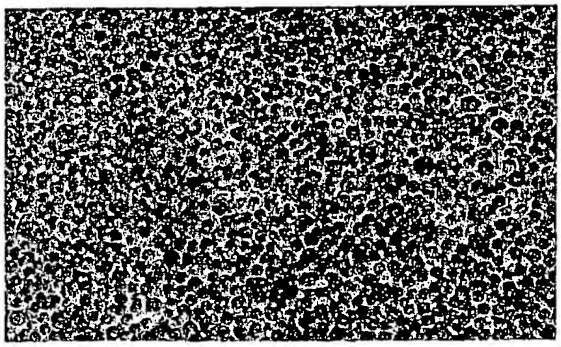
L3



L6



L4



SFV

FIGURE 2 (CON'T)

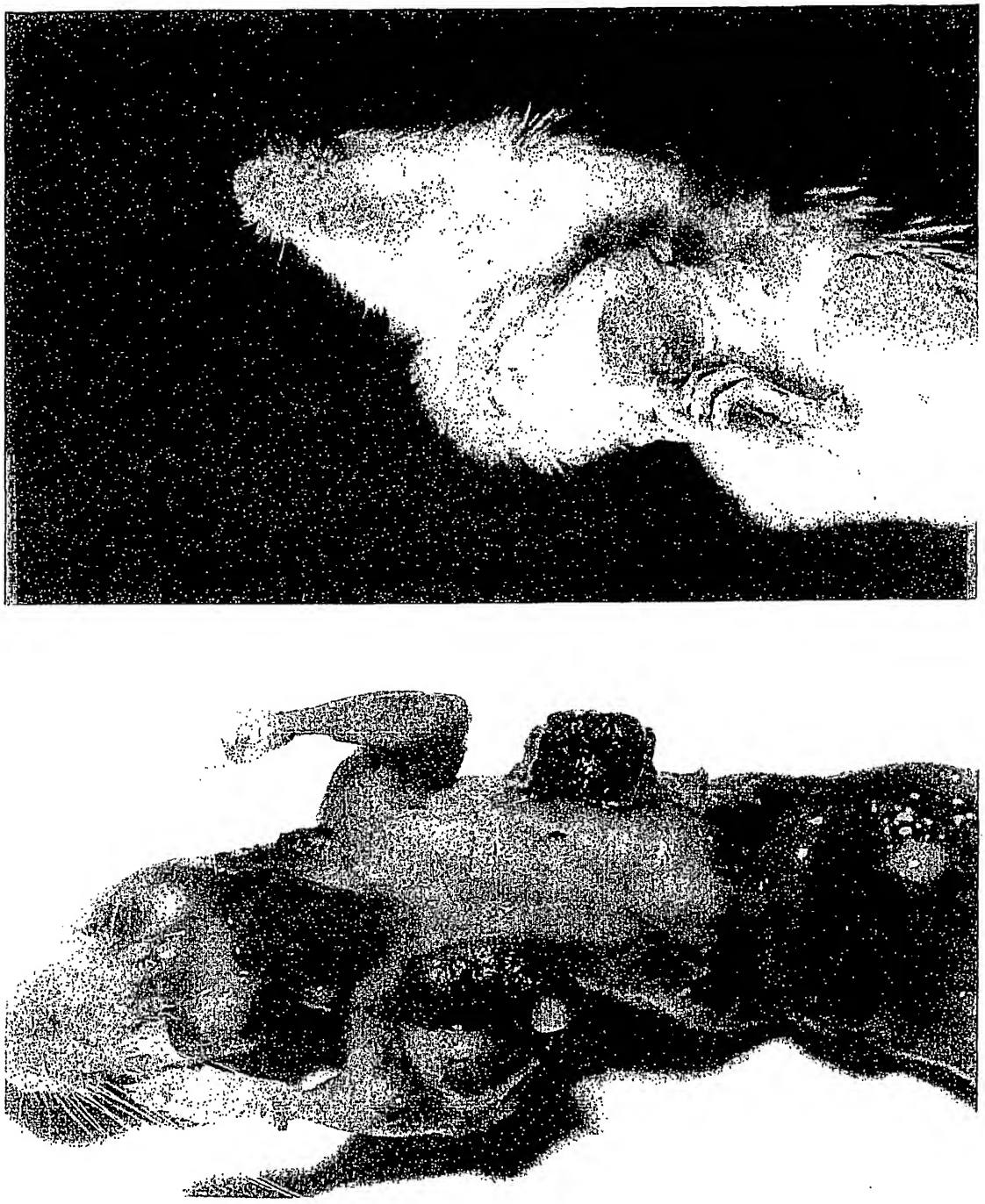


FIGURE 3A

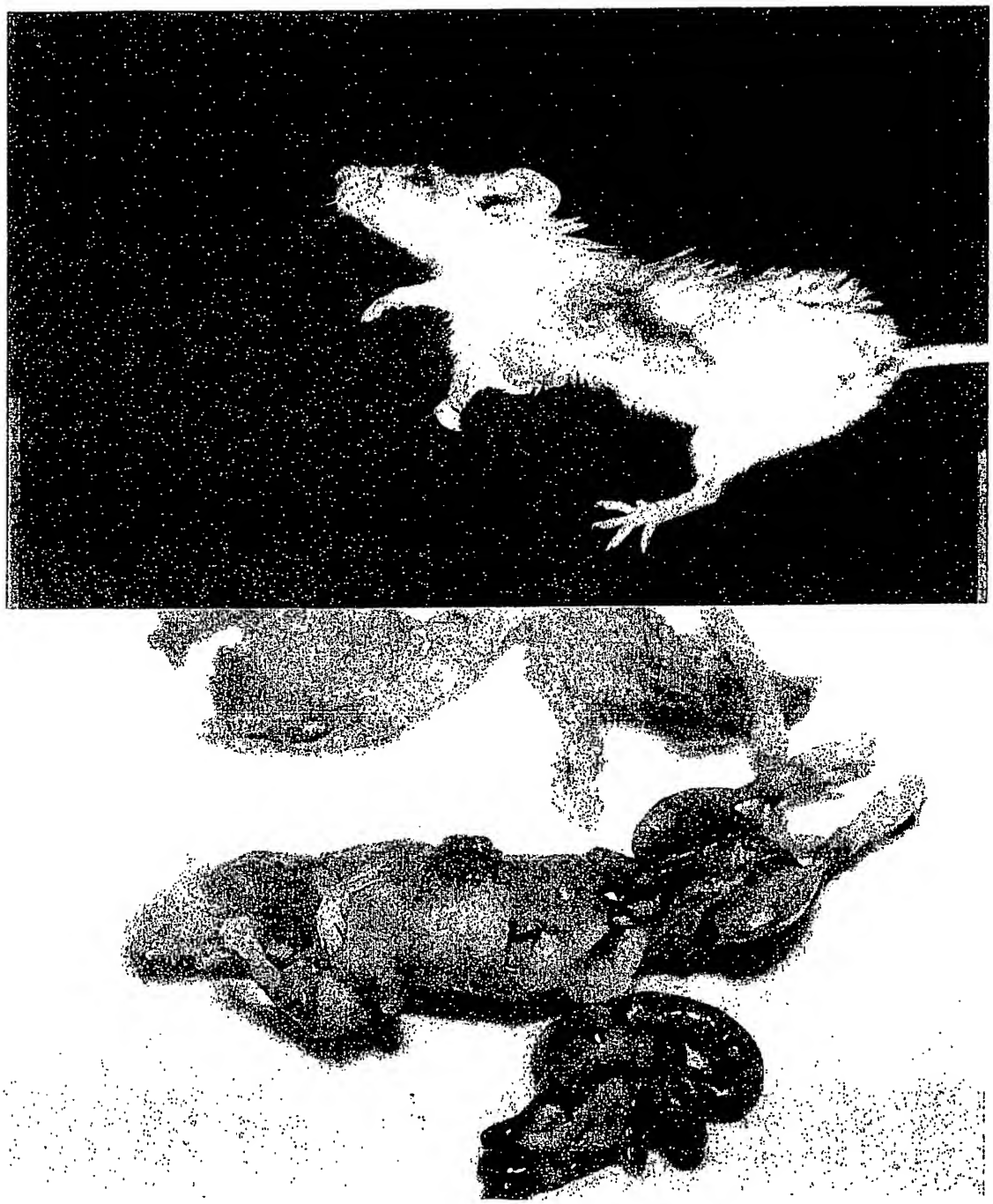


FIGURE 3B

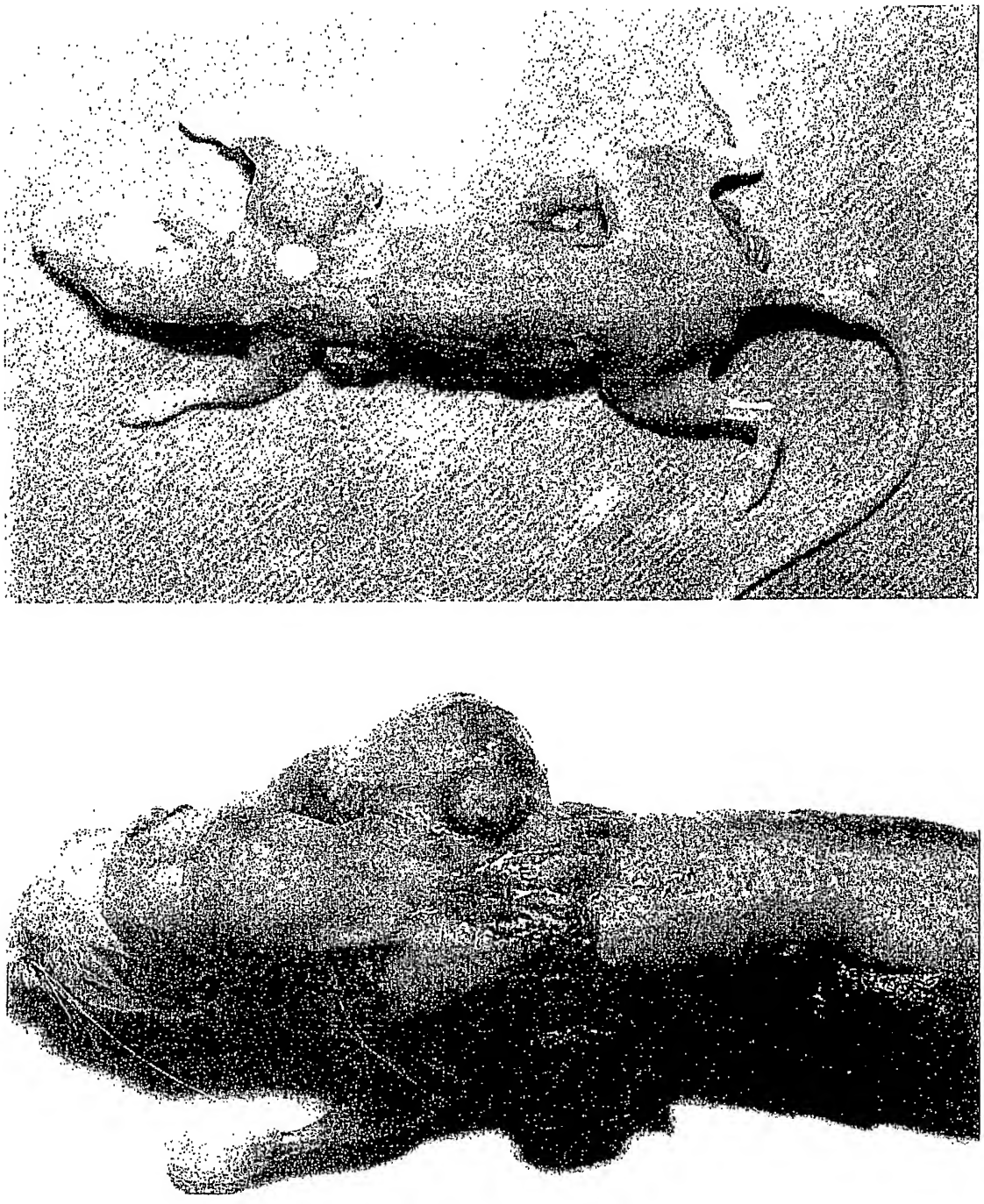


FIGURE 3C

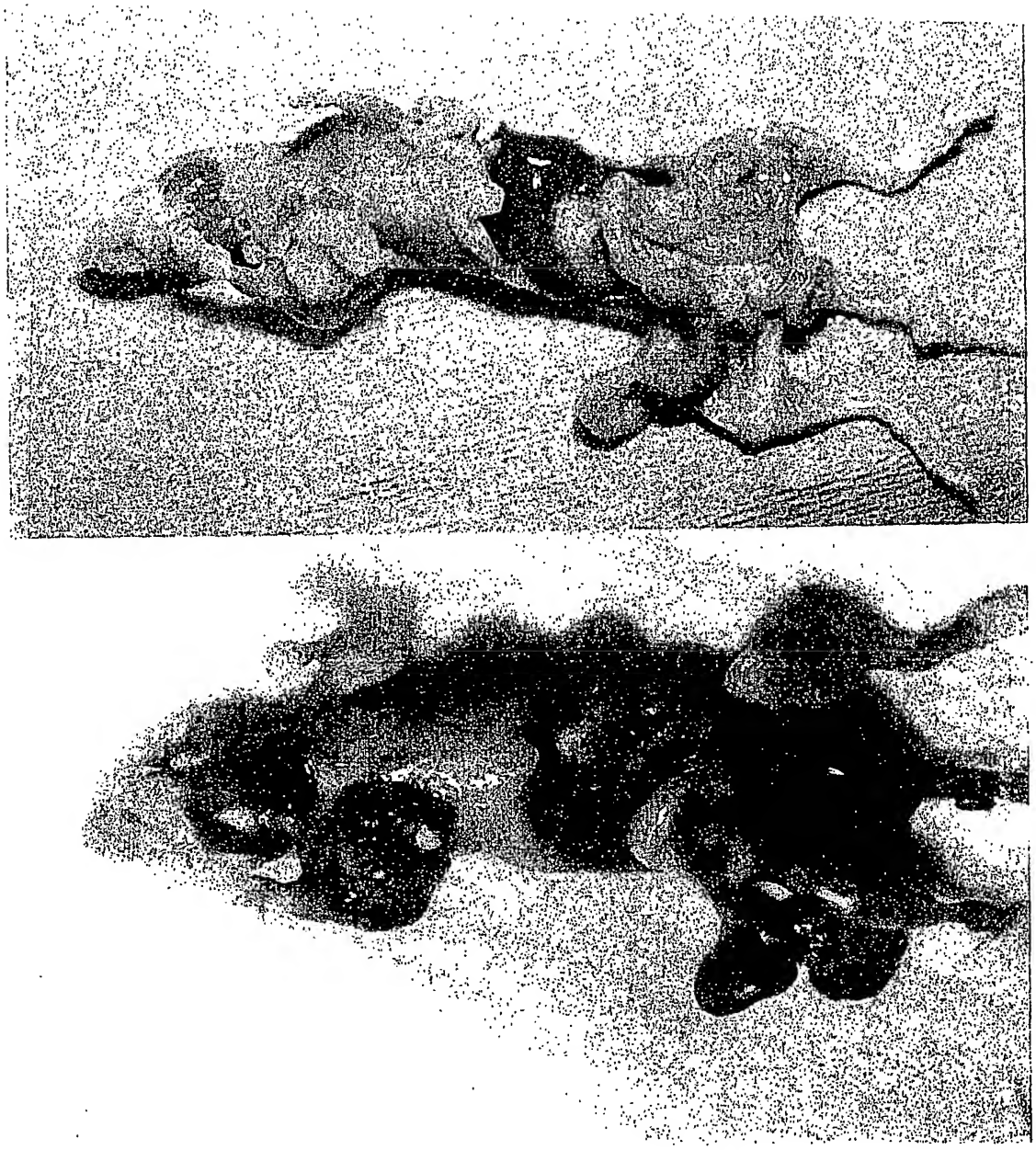


FIGURE 3D

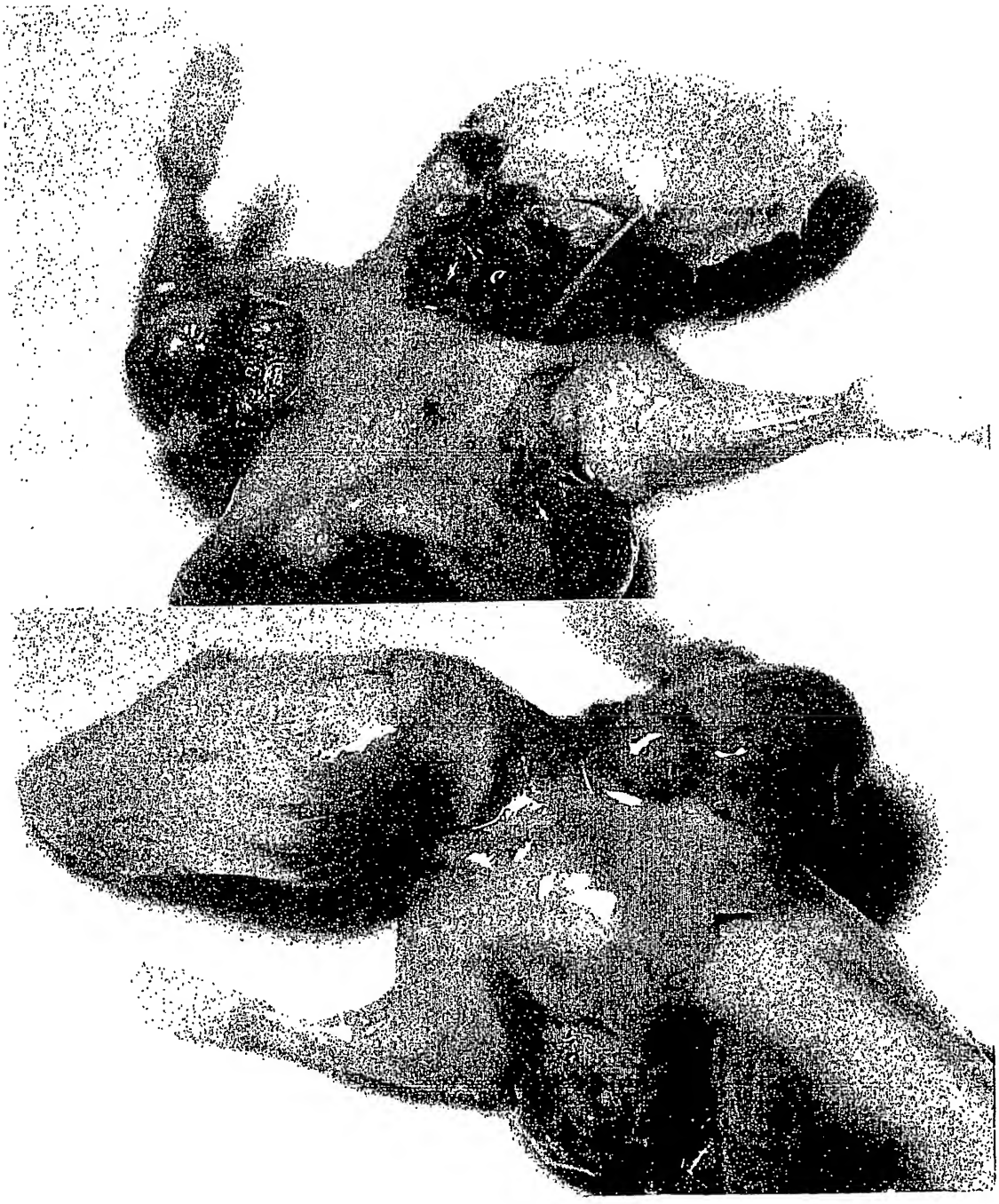


FIGURE 3E

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(74) Agents: **KONSKI, Antoinette, F.** et al.; Mc Cutchen, Doyle, Brown & Enersen, LLP., Three Embarcaderro Center, Suite 1800, San Francisco, CA 94111-4067 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

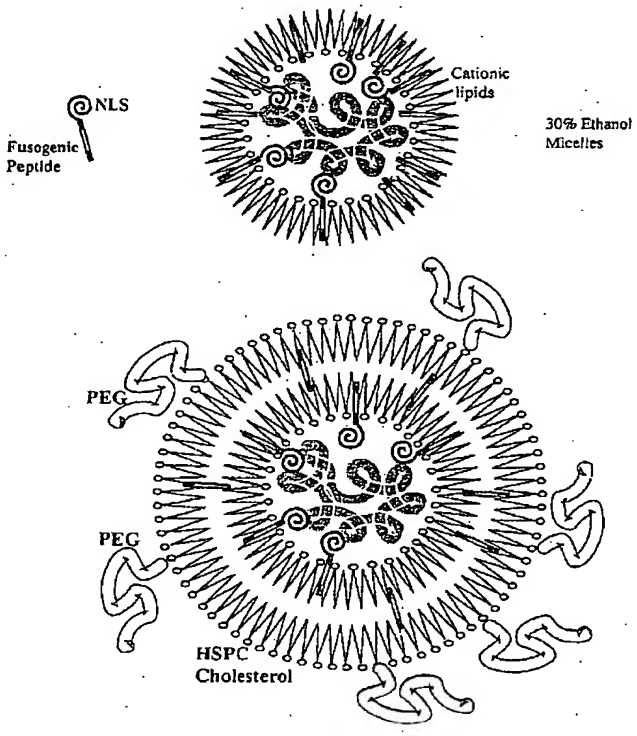
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
3 October 2002

[Continued on next page]

(54) Title: ENCAPSULATION OF POLYNUCLEOTIDES AND DRUGS INTO TARGETED LIPOSOMES



(57) Abstract: A method is disclosed for encapsulating plasmids, oligonucleotides or negatively-charged drugs into liposomes having a different lipid composition between their inner and outer membrane bilayers and able to reach primary tumors and their metastases after intravenous injection to animals and humans. The formulation method includes complex formation between DNA with cationic lipid molecules and fusogenic/NLS peptide conjugates composed of a hydrophobic chain of about 10-20 amino acids and also containing four or more histidine residues or NLS at their one end. The encapsulated molecules display therapeutic efficacy in eradicating a variety of solid human tumors including but not limited to breast carcinoma and prostate carcinoma. Combination of the plasmids, oligonucleotides or negatively-charged drugs with other anti-neoplastic drugs (the positively-charged cis-platin, doxorubicin) encapsulated into liposomes are of therapeutic value. Also of therapeutic value in cancer eradication are combinations of encapsulated the plasmids, oligonucleotides or negatively-charged drugs with HSV-tk plus encapsulated ganciclovir.

WO 01/093836 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/18657

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 C12N15/88 A61K9/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 635 487 A (WOLFF JON A ET AL) 3 June 1997 (1997-06-03) column 28 -column 29; example 4	1-5,30, 34,37,38
X	WO 99 29303 A (EXPRESSION GENETICS INC) 17 June 1999 (1999-06-17) page 8, line 5 - line 6	30,34, 37,38
X	WO 97 11682 A (UNIV PITTSBURGH) 3 April 1997 (1997-04-03) examples page 4, line 21 - line 30	30,34, 37,38

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2002	29/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Boulois, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SIMOES S ET AL: "Enhancement of cationic liposome-mediated gene delivery by transferrin and fusogenic peptides" PROCEEDINGS OF THE 24TH. INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE OF BIOACTIVE MATERIALS. STOCKHOLM, JUNE 15 - 19, 1997, PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE OF BIOACTIVE MATERIALS, DEERFIELD, IL., CONTROLLED RELEASE , vol. SYMP. 24, 15 June 1997 (1997-06-15), pages 659-660, XP002098090 ISSN: 1022-0178 the whole document	31-33
X	CHAVEZ A ET AL: "PH-INDUCED DESTABILIZATION OF LIPID BILAYERS BY A PEPTIDE FROM THE VP3 PROTEIN OF THE CAPSID OF HEPATITIS A VIRUS" ANALYST, LONDON, GB, vol. 11, no. 123, November 1998 (1998-11), pages 2251-2256, XP008002817 the whole document	31-33
X	ARONSOHN A I ET AL: "NUCLEAR LOCALIZATION SIGNAL PEPTIDES ENHANCE CATIONIC LIPOSOME-MEDIATED GENE THERAPY" JOURNAL OF DRUG TARGETING, HARWOOD ACADEMIC PUBLISHERS GMBH, DE, vol. 3, no. 5, 1998, pages 163-169, XP008002709 ISSN: 1061-186X the whole document	31-33
X	PEDROSO DE LIMA M C ET AL: "GENE DELIVERY MEDIATED BY CATIONIC LIPOSOMES: FROM BIOPHYSICAL ASPECTS TO ENCHANCEMENT OF TRANSFECTION" MOLECULAR MEMBRANE BIOLOGY, TAYLOR AND FRANCIS, GB, vol. 16, no. 1, 1999, pages 103-109, XP001030617 ISSN: 0968-7688 the whole document	31-33

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/18657

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	BOULIKAS T ET AL: "HISTONES, PROTAMINE, AND POLYLYSINE BUT NOT POLY(E:K) ENHANCE TRANSFECTION EFFICIENCY" INTERNATIONAL JOURNAL OF ONCOLOGY, EDITORIAL ACADEMY OF THE INTERNATIONAL JOURNAL OF ONCOLOGY,, GR, vol. 10, no. 2, 1 February 1997 (1997-02-01), pages 317-322, XP002058322 ISSN: 1019-6439 the whole document	31-33

INTERNATIONAL SEARCH REPORT

ion on patent family members

International Application No
PCT/US 01/18657

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5635487 A	03-06-1997	NONE	
WO 9929303 A	17-06-1999	AU 740342 B2	01-11-2001
		AU 1910899 A	28-06-1999
		BR 9813548 A	10-10-2000
		CN 1281355 T	24-01-2001
		EP 1037611 A1	27-09-2000
		JP 2001525357 T	11-12-2001
		WO 9929303 A1	17-06-1999
		US 6210717 B1	03-04-2001
		ZA 9811376 A	28-06-1999
WO 9711682 A	03-04-1997	US 6120794 A	19-09-2000
		AU 721245 B2	29-06-2000
		AU 7245896 A	17-04-1997
		CA 2230940 A1	03-04-1997
		EP 0852490 A2	15-07-1998
		JP 11512712 T	02-11-1999
		WO 9711682 A2	03-04-1997

The search profile you entered was too complex or gave too many answers. Simplify or subdivide the query and try again. If you have exceeded the answer limit, enter DELETE HISTORY at an arrow prompt (=>) to remove all previous answers sets and begin at L1. Use the SAVE command to store any important profiles or answer sets before using DELETE HISTORY.

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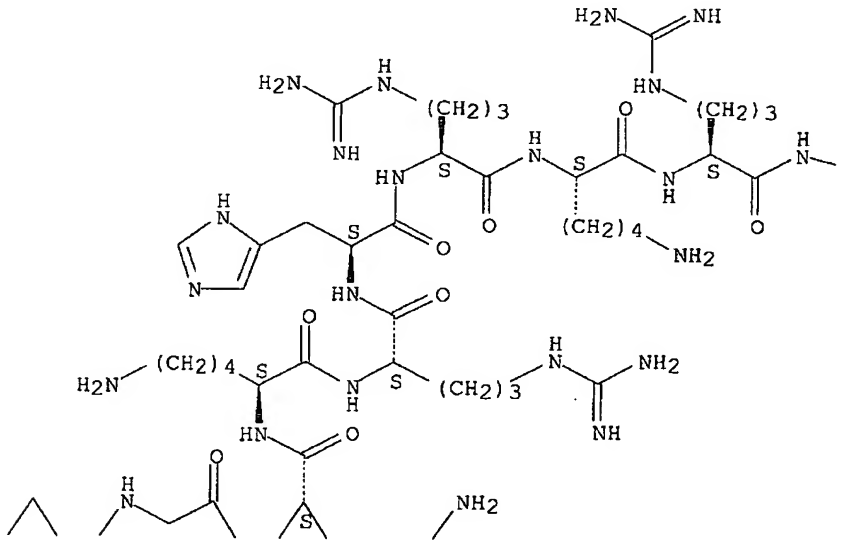

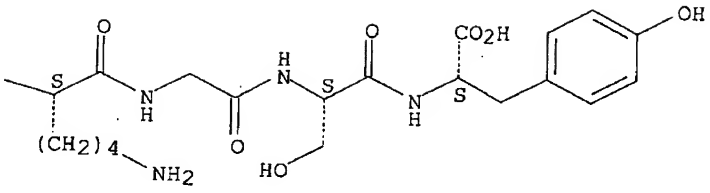

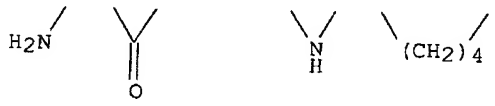

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CN 576: PN: WO0193836 SEQID: 574 claimed protein
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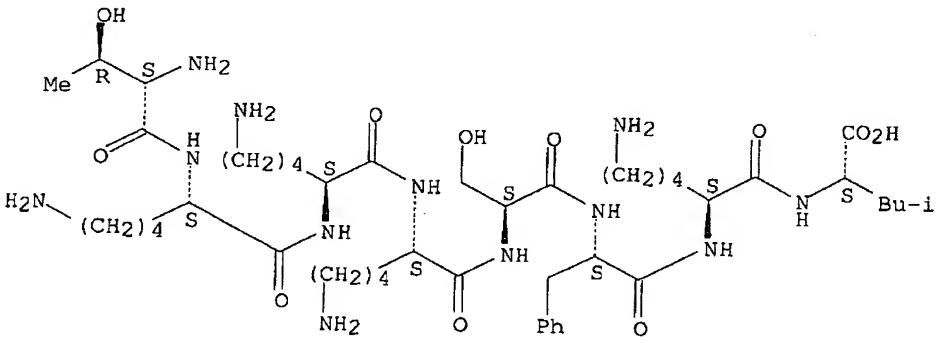
Absolute stereochemistry.



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CN 547: PN: WO0193836 SEQID: 545 claimed protein
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Absolute stereochemistry.

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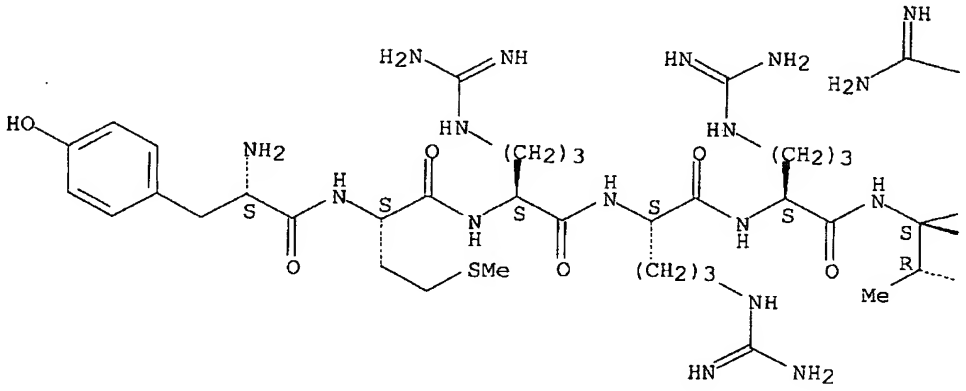

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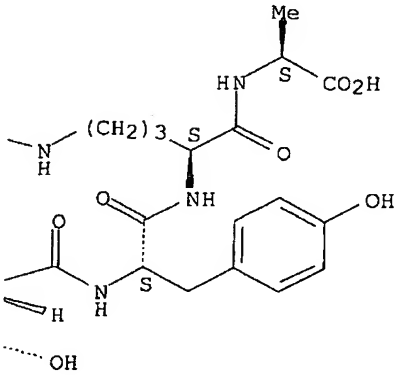

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Absolute stereochemistry.

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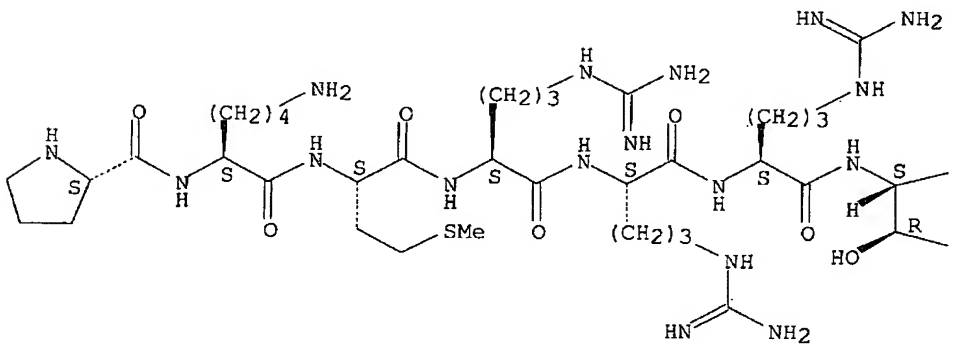

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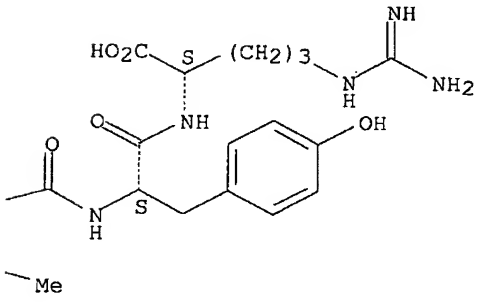

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LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

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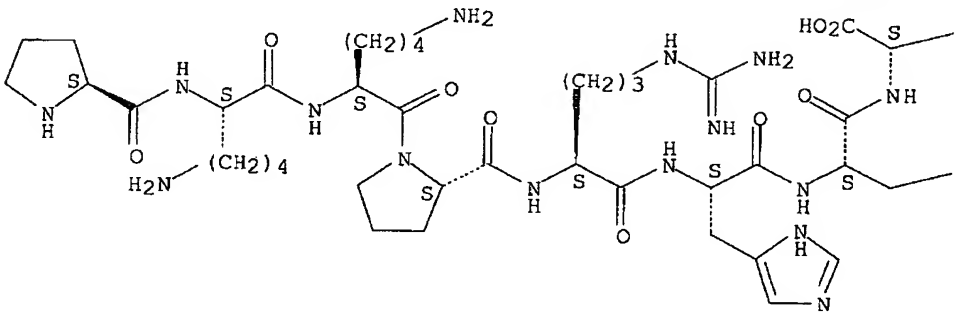

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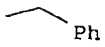

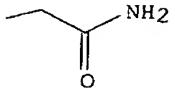

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Absolute stereochemistry.

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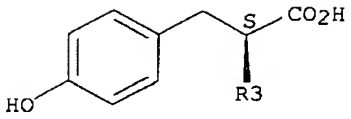

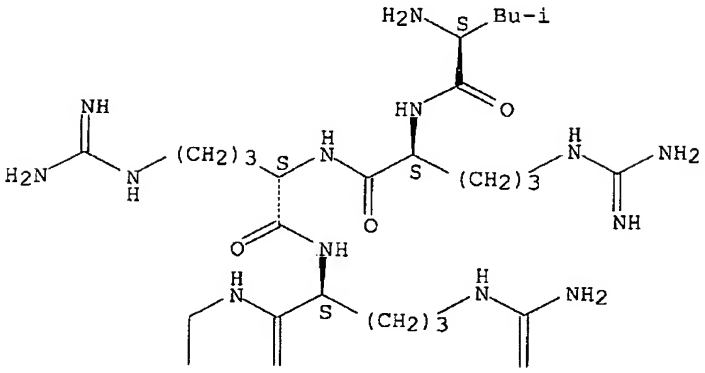

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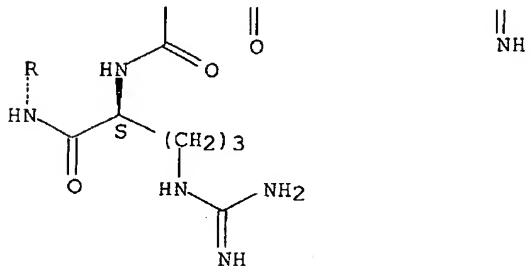

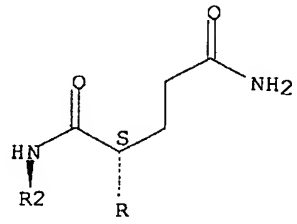

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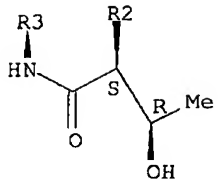

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Absolute stereochemistry.

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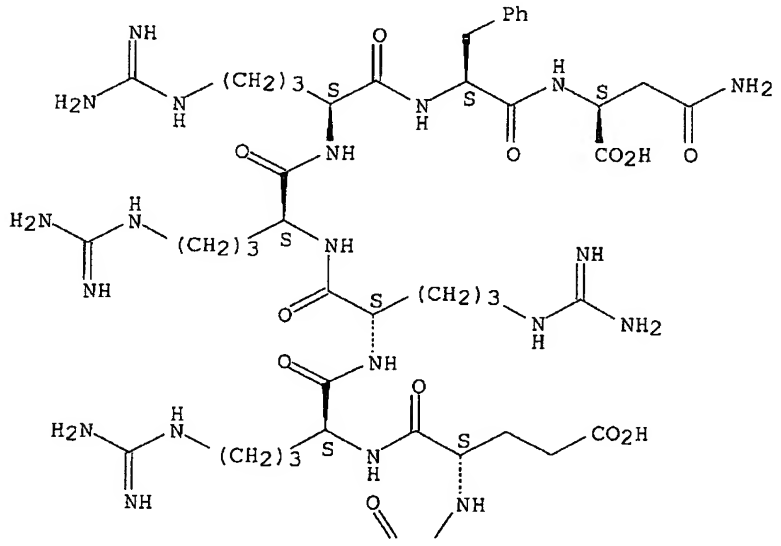

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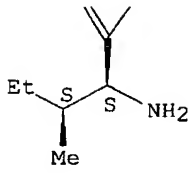

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Absolute stereochemistry.

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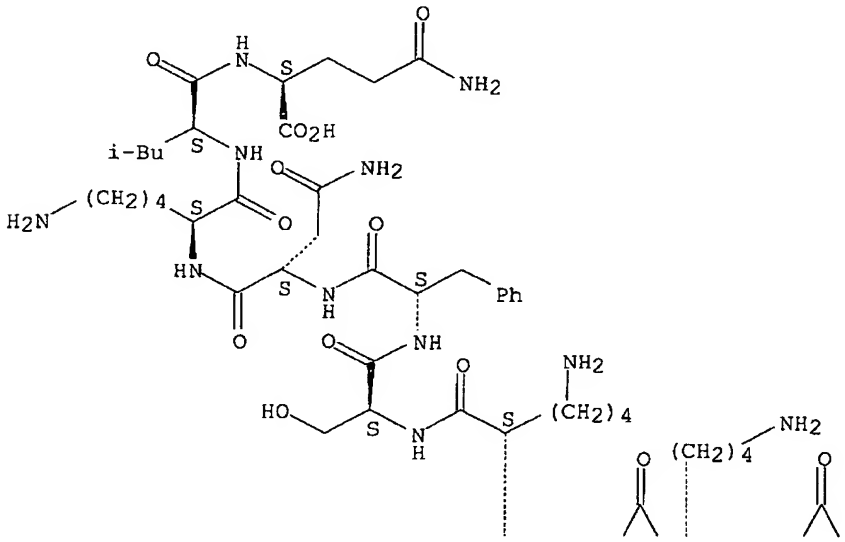

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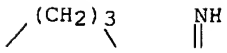

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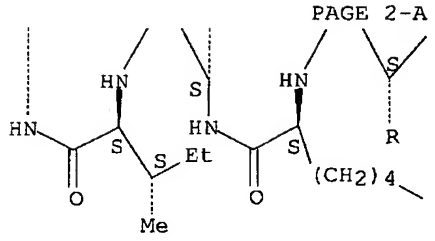

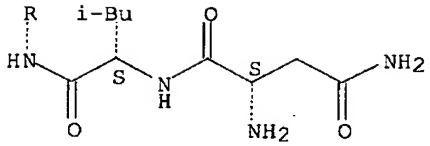

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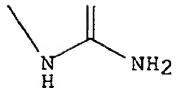

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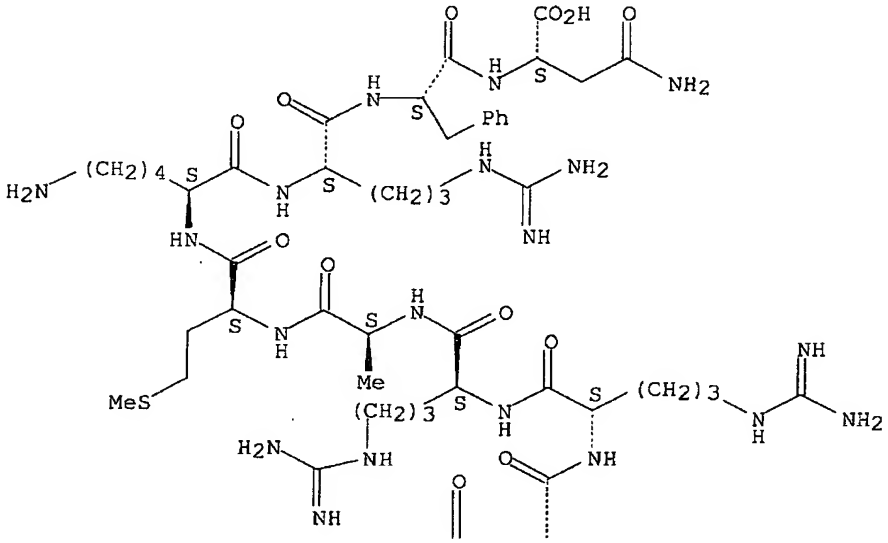
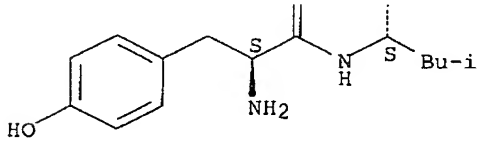
CN L-Asparagine, L-tyrosyl-L-leucyl-L-arginyl-L-arginyl-L-alanyl-L-methionyl-L-lysyl-L-arginyl-L-phenylalanyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 231: PN: WO0193836 SEQID: 229 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C60 H99 N21 O13 S
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



PAGE 2-A



PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

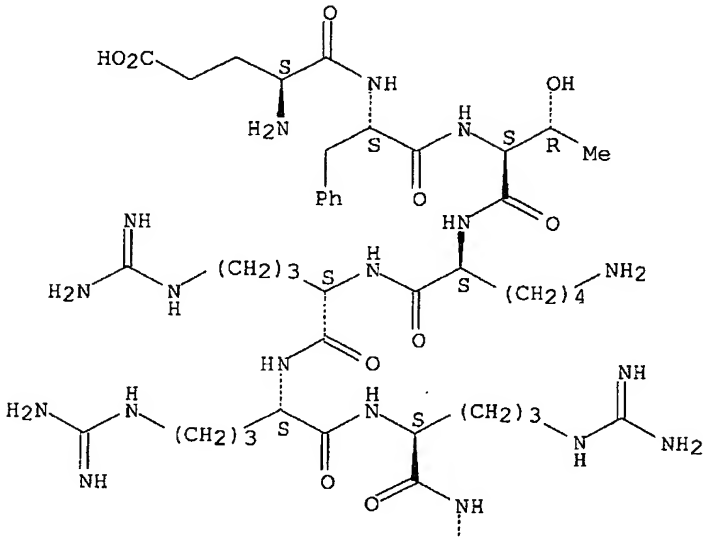
1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 10 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379719-05-8 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Leucine, L-α-glutamyl-L-phenylalanyl-L-threonyl-L-lysyl-L-arginyl-L-arginyl-L-arginyl-L-threonyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 218: PN: WO0193836 SEQID: 216 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH

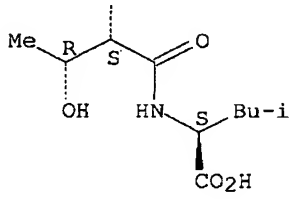
MF C52 H91 N19 O14
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



PAGE 2-A



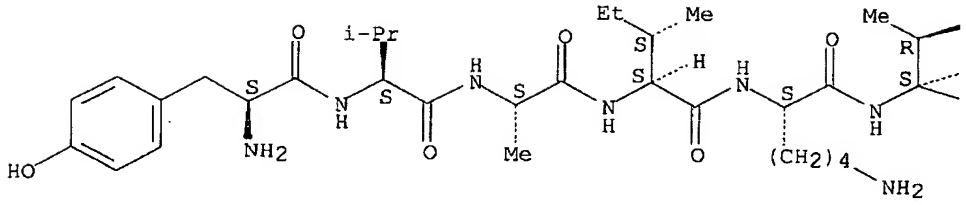
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

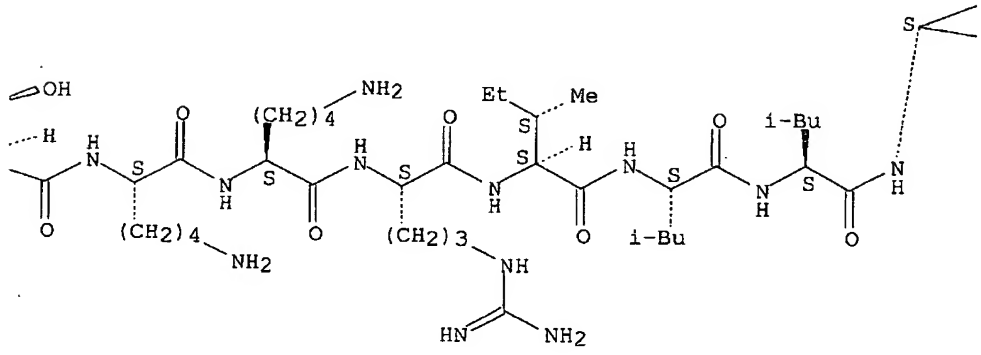
L3 ANSWER 11 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379719-02-5 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Methionine, L-tyrosyl-L-valyl-L-alanyl-L-isoleucyl-L-lysyl-L-threonyl-L-lysyl-L-lysyl-L-arginyl-L-isoleucyl-L-leucyl-L-leucyl-L-tyrosyl-L-threonyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 215: PN: WO0193836 SEQID: 213 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C87 H149 N21 O20 S
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

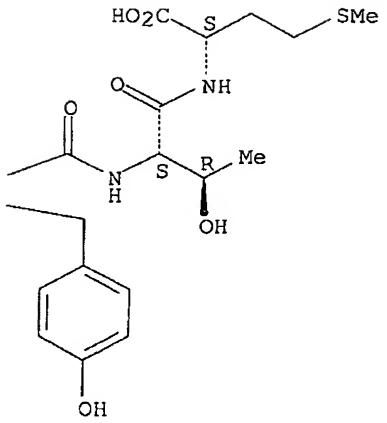
PAGE 1-A



PAGE 1-B



PAGE 1-C



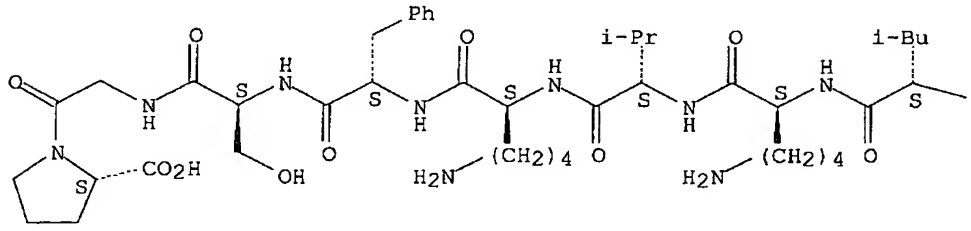
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 12 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379718-85-1 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Proline, L-lysyl-L-tyrosyl-L-alanyl-L-valyl-L-lysyl-L-lysyl-L-leucyl-L-lysyl-L-valyl-L-lysyl-L-phenylalanyl-L-serylglycyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 196: PN: WO0193836 SEQID: 194 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C77 H129 N19 O17
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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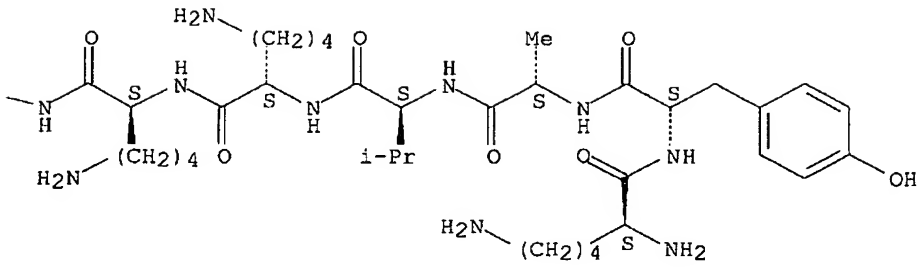


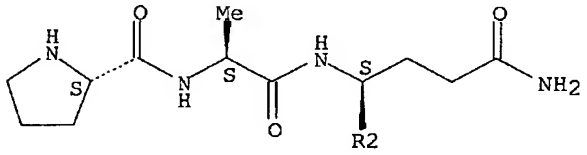
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 13 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379718-83-9 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Aspartic acid, L-prolyl-L-alanyl-L-glutaminyl-L-lysyl-L-leucyl-L-arginyl-L-lysyl-L-lysyl-L-asparaginyl-L-asparaginyl-L-phenylalanyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 194: PN: WO0193836 SEQID: 192 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C64 H107 N21 O18
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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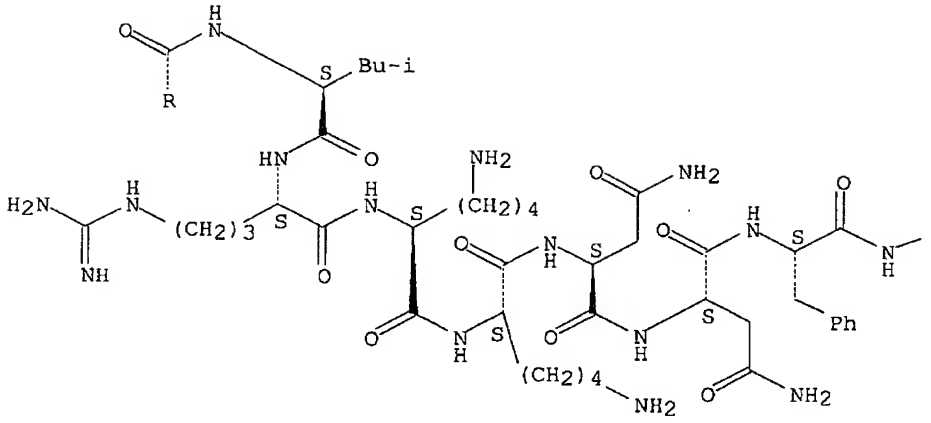
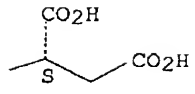


PAGE 2-B



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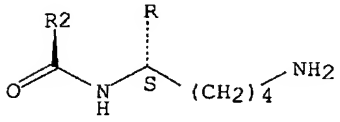


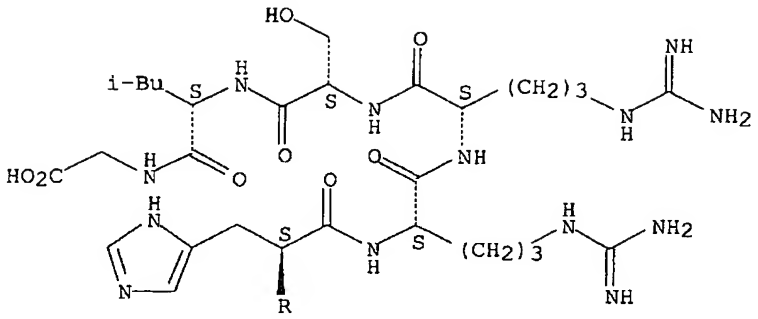
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 14 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379718-51-1 REGISTRY
ED Entered STN: 31 Dec 2001
CN Glycine, L-α-glutamyl-L-leucyl-L-arginyl-L-glutaminyl-L-phenylalanyl-L-histidyl-L-arginyl-L-arginyl-L-seryl-L-leucyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 159: PN: WO0193836 SEQID: 157 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C60 H99 N23 O16
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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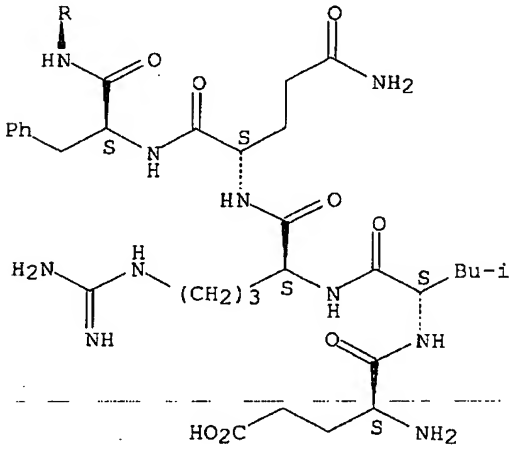


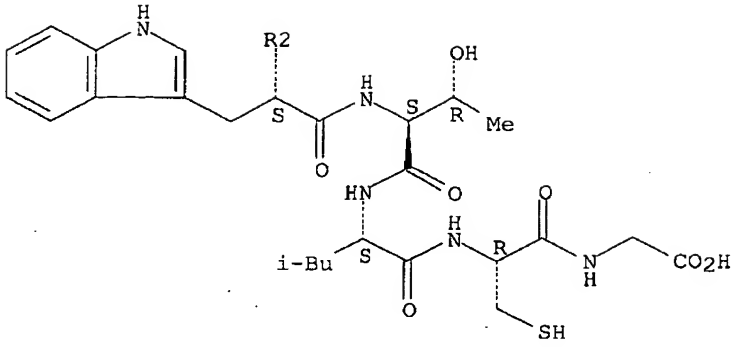
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 15 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379718-38-4 REGISTRY
ED Entered STN: 31 Dec 2001
CN Glycine, glycyl-L-phenylalanyl-L-alanyl-L-lysyl-L-arginyl-L-valyl-L-lysylglycyl-L-arginyl-L-threonyl-L-tryptophyl-L-threonyl-L-leucyl-L-cysteinyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 142: PN: WO0193836 SEQID: 140 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C75 H122 N24 O18 S
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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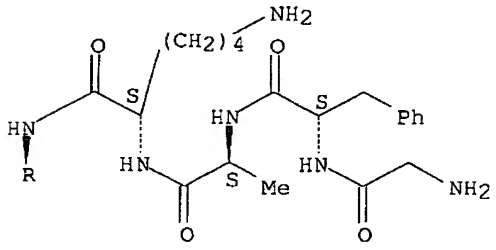

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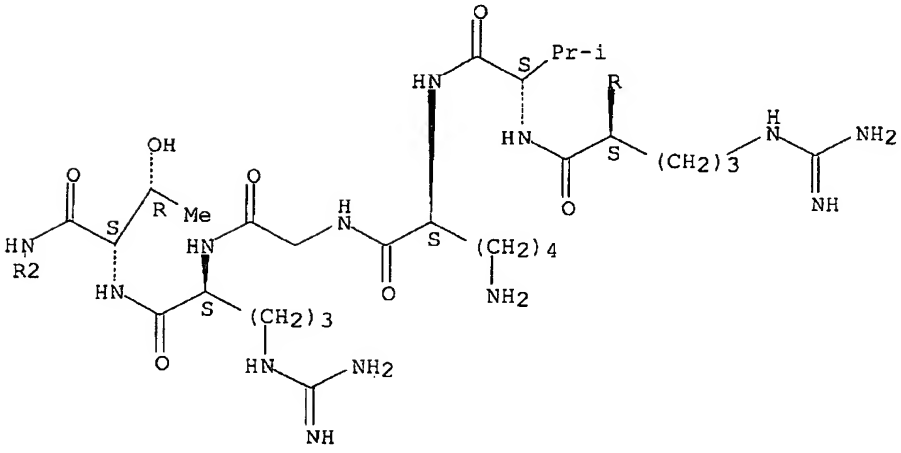


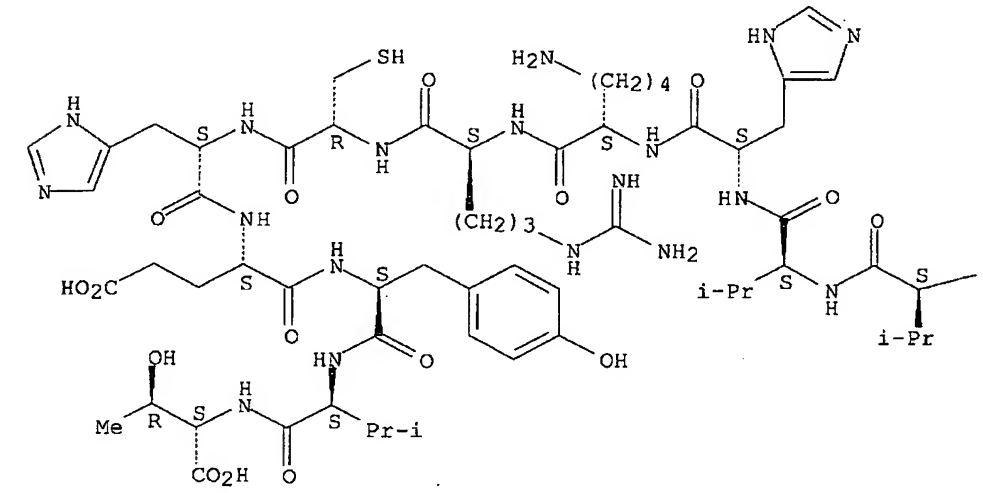
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 16 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379718-30-6 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Threonine, L-seryl-L-tyrosyl-L-valyl-L-valyl-L-histidyl-L-lysyl-L-arginyl-L-cysteinyl-L-histidyl-L-α-glutamyl-L-tyrosyl-L-valyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 132: PN: WO0193836 SEQID: 130 claimed protein
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C72 H109 N21 O20 S
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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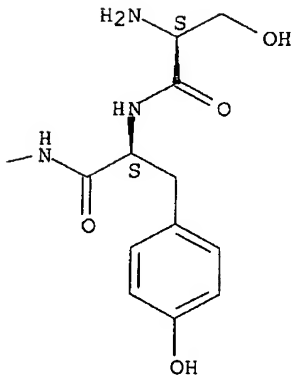


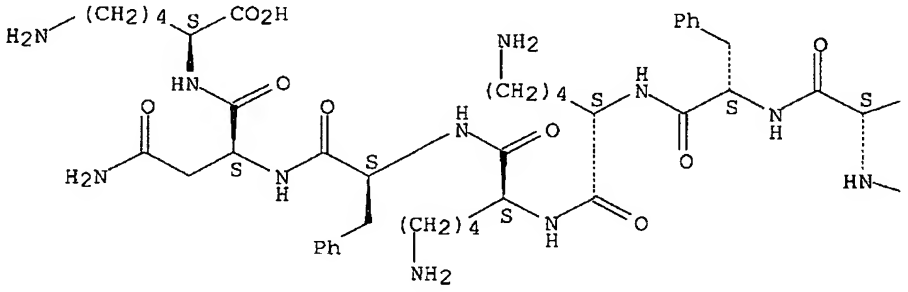
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 17 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379717-85-8 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Lysine, L-arginyl-L-lysyl-L-phenylalanyl-L-lysyl-L-lysyl-L-phenylalanyl-L-asparaginyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 58: PN: WO0193836 SEQID: 56 claimed protein
CN 66: PN: WO2006042214 SEQID: 30 unclaimed sequence
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C52 H86 N16 O10
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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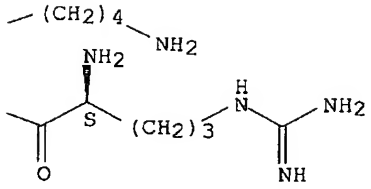


PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

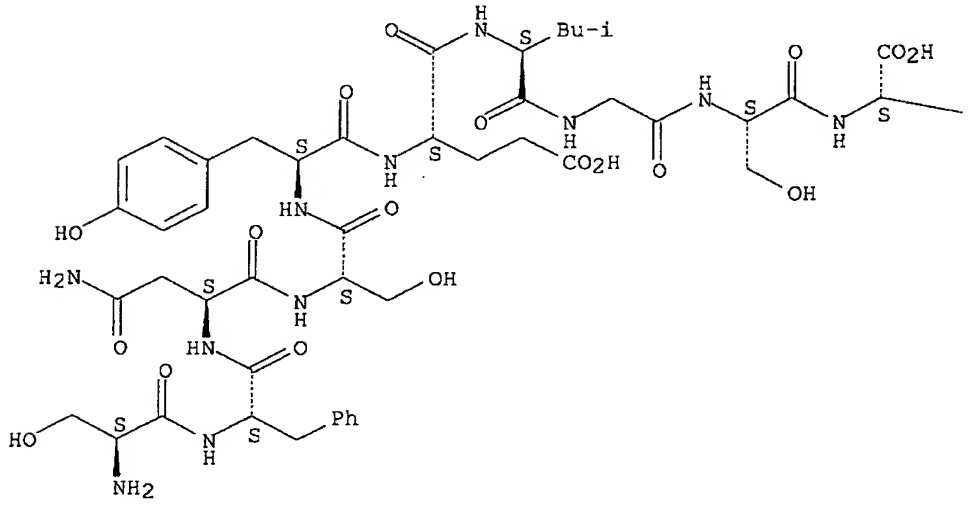
2 REFERENCES IN FILE CA (1907 TO DATE)
2 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 18 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379711-25-8 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Leucine, L-seryl-L-phenylalanyl-L-asparaginyl-L-seryl-L-tyrosyl-L-α-glutamyl-L-leucylglycyl-L-seryl- (CA INDEX NAME)
OTHER NAMES:
CN 15: PN: US20060148700 SEQID: 15 claimed protein
CN 1: PN: US20060148702 SEQID: 1 claimed sequence

CN 1: PN: WO2005099721 SEQID: 1 claimed protein
CN 1: PN: WO2006080941 SEQID: 1 claimed sequence
CN 1: PN: WO2007027974 SEQID: 1 claimed protein
CN 33: PN: US20060153867 SEQID: 34 claimed sequence
CN 3: PN: WO2005107789 SEQID: 3 claimed sequence
CN 4: PN: WO2007143119 SEQID: 4 unclaimed sequence
CN 8-17-δ protein kinase C (Rattus norvegicus isoform δV1-1) (Rattus norvegicus)
CN 8-17-Kinase (phosphorylating), protein, nPKC (Rattus norvegicus)
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C50 H73 N11 O18
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER, USPAT2, USPATFULL

Absolute stereochemistry.

PAGE 1-A



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—Bu-i

PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

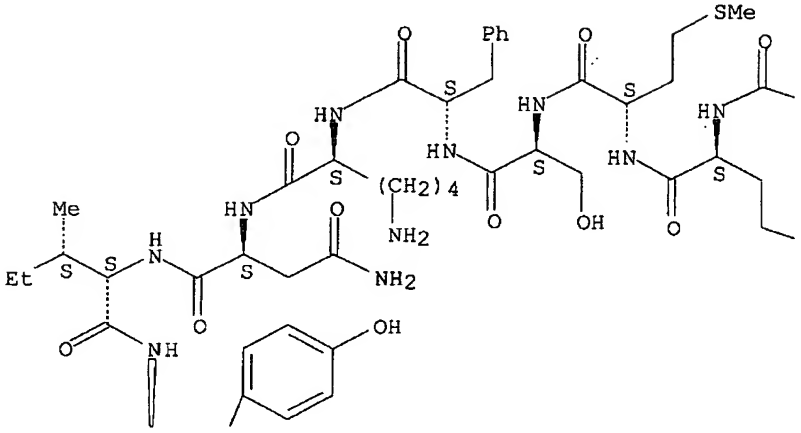
15 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
15 REFERENCES IN FILE CAPLUS (1907 TO DATE)

L3 ANSWER 19 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379705-46-1 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Tyrosine, L-methionyl-L-α-glutamyl-L-cysteinylglycyl-L-glutaminyl-L-methionyl-L-seryl-L-phenylalanyl-L-lysyl-L-asparaginyl-L-isoleucyl-L-tyrosyl-L-histidyl-L-lysyl- (9CI) (CA INDEX NAME)
OTHER NAMES:

CN 9: PN: WO0192328 SEQID: 7 unclaimed sequence
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C83 H123 N21 O23 S3
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER

Absolute stereochemistry.

PAGE 1-A



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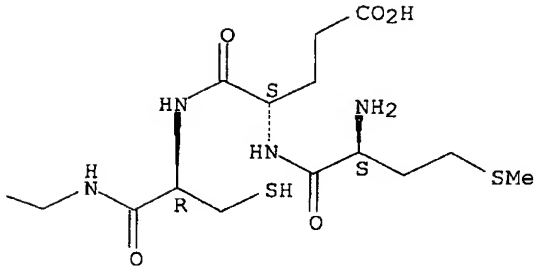

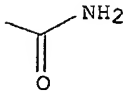

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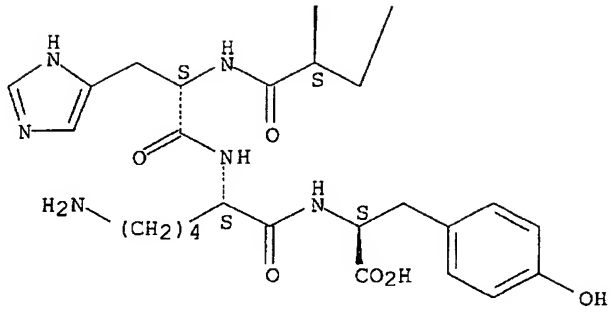


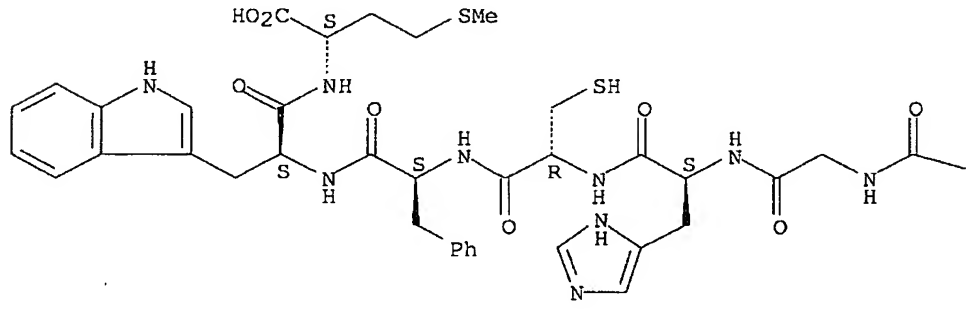
PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

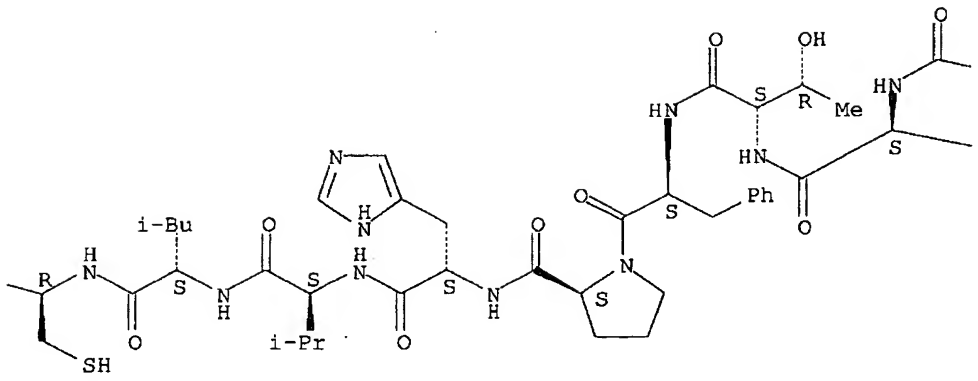
L3 ANSWER 20 OF 16945 REGISTRY COPYRIGHT 2008 ACS on STN
RN 379700-35-3 REGISTRY
ED Entered STN: 31 Dec 2001
CN L-Methionine, L-methionyl-L-α-aspartyl-L-threonyl-L-phenylalanyl-L-prolyl-L-histidyl-L-valyl-L-leucyl-L-cysteinylglycyl-L-histidyl-L-cysteinyl-L-phenylalanyl-L-tryptophyl- (9CI) (CA INDEX NAME)
OTHER NAMES:
CN 1: PN: WO0192517 SEQID: 7 unclaimed sequence
FS PROTEIN SEQUENCE; STEREOSEARCH
MF C83 H114 N20 O19 S4
SR CA
LC STN Files: CA, CAPLUS, TOXCENTER

Absolute stereochemistry.

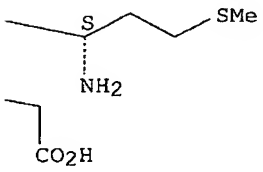
PAGE 1-A



PAGE 1-B



PAGE 1-C



PROPERTY DATA AVAILABLE IN THE 'PROP' FORMAT

1 REFERENCES IN FILE CA (1907 TO DATE)
1 REFERENCES IN FILE CAPLUS (1907 TO DATE)

=> logh
LOGH IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> log h

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	86.22	86.43

SESSION WILL BE HELD FOR 120 MINUTES
STN INTERNATIONAL SESSION SUSPENDED AT 22:02:51 ON 20 FEB 2008